

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
23 May 2002 (23.05.2002)

PCT

(10) International Publication Number  
**WO 02/40059 A2**

(51) International Patent Classification<sup>7</sup>: **A61K 48/00**

(21) International Application Number: PCT/US01/45626

(22) International Filing Date:  
1 November 2001 (01.11.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
09/704,232 1 November 2000 (01.11.2000) US

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(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published:**

— without international search report and to be republished upon receipt of that report

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

(54) Title: METHODS AND COMPOSITIONS FOR INDUCING CELL-MEDIATED IMMUNE RESPONSES

(57) Abstract: The invention provides methods and compositions for treating a subject having undesired cells, e.g., tumor cells or virally infected cells. The method comprises, e.g., administering a polynucleotide encoding a variant of an antigen present on the undesired cell ("target cell"), which variant is not secreted by, or expressed on the extracellular side of the cell membrane of, the cell in which it is expressed. The variant is preferably biologically inactive. The invention provides a method for inducing a cell-mediated immune response against the undesired cell without inducing a humoral immune response. In addition, the method is safe, since the absence of a biological activity of the variant prevents the occurrence of deleterious effects in the subject receiving the variant.



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## METHODS AND COMPOSITIONS FOR INDUCING CELL-MEDIATED IMMUNE RESPONSES

### Background of the Invention

5 All normal human nucleated cells express on their membrane small protein fragments derived from de novo protein synthesis. These so-called peptides are associated with the major histocompatibility complex (MHC) class I molecules and form the antigens which are recognized by CD8 cytotoxic T-lymphocytes (CTLs). Such recognition is important for the elimination of virally infected cells, of tumor cells, or of cells that contain  
10 intracellular parasites. For this to occur, potentially antigen-reactive T cells need to be "pre-educated" by recognizing the antigen in question on the membrane of professional antigen-presenting cells (APCs), such as dendritic cells (DCs) which, in addition to the antigen, provide co-stimulatory "maturation" signals to the T cells. In the absence of such signals the T cells become paralyzed and tolerant to the antigens in question.

15 Recent studies have shown that tumor cells are poor APCs, and that "professional" antigen presenting cells are essential for induction of CD4+ and CD8+ T cell responses against tumors. In particular, Huang, A. Y. C., et al., Science (Wash. DC) 264:961 (1994), reported that the *in vivo* priming of MHC class I-restricted responses involves a transfer of tumor antigens to a host bone marrow-derived dendritic cell ("IBM-DC") and subsequent  
20 presentation to CD8+T cell effectors. A correlation between the number of dendritic cells ("DC") infiltrating a cancer and longer patient survival or reduced frequency of metastatic disease have been observed for a variety of cancers. See, Becker, Y., Vivo 7:187 (1993). Thus, for the generation of an immune response against a tumor, the tumor antigen(s) need(s) to be expressed by professional APCs. This presentation has been accomplished by  
25 *in vitro* exposure of dendritic cells to either tumor lysates that presumably contain tumor antigens, to purified tumor antigens, or to peptides derived from such antigens.

For example, U.S. Patent 5,788,963 discloses the use of human dendritic cells to activate T cells for immuno-therapeutic response against primary and metastatic prostate cancer. Human dendritic cells are isolated and exposed to PSMA-derived peptides *in vitro*.  
30 The PSMA-derived peptides exchange with peptides already bound to MHC molecules on the dendritic cells, enabling them to stimulate killer cells which then lyse prostate cells. A major problem with this technique comes from the low efficiency of peptide exchange and

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from the necessity to work with different immunodominant peptides for patients with different MHC phenotypes. In addition, since the function of such tumor-associated or tissue-specific antigens is largely unknown, their synthesis and release by patients' cells *in vivo*, may lead to serious side effects. Furthermore, in cases where dendritic cells are transfected *in vitro*, expression of a functional protein may alter the dendritic cells viability, change their migration pattern or their ability to provide co stimulation to T cells.

Other methods for treating cancer include administering to a patient antibodies directed against a surface antigen of the tumor cells, wherein the antibodies are conjugated with a cytotoxic agent. For example, U.S. Patent 5,227,471 discloses a method for treating prostate cancer which involves an antibody directed against the prostate-specific membrane antigen and a cytotoxic agent conjugated thereto. However, since the PSMA is expressed on normal brain cells, use of antibodies which may transverse through the blood-brain barrier and may damage normal brain cells may not be permitted.

Thus, there is a need for a safe and efficient method for eliminating undesired cells, e.g., cancer cells, which is inactive against most normal cells.

### Summary of the Invention

The invention provides methods and compositions for inducing a cell-mediated immune response against a target antigen in a subject, without inducing a humoral immune response against that target antigen. Generally, the invention provides methods for eliminating undesired cells (referred to herein as "target cells") in a subject (*in vivo*) or *in vitro* (e.g., *ex vivo*). In a preferred embodiment of the invention, the method comprises administering to the subject a polynucleotide encoding a polypeptide which is a variant of an antigen that is expressed in a target cell, which target antigen is then presented within the context of MHC class I on the surface of the target cells such that a cell-mediated immune response against the cell comprising the target antigen develops; and wherein the variant of the target antigen is not secreted from, nor expressed on the extracellular side of the cell membrane of a cell in which it is synthesized. In an even more preferred embodiment, the variant of the target antigen is biologically inactive, so that the survival and the function of cells in which the said antigen is expressed, is not altered.

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In one embodiment, the undesired cells are tumor cells, and the target antigen is a tumor antigen, e.g., an antigen that is expressed preferentially on tumor cells. In a preferred embodiment, the undesired cells are prostate tumor cells and the target antigen is prostate specific membrane antigen (PSMA), prostate specific antigen (PSA) or prostate acidic phasphatase (PAP). In an even more preferred embodiment, the variant of the PSMA antigen comprises amino acids 44-750; 55-750; 61-750; 76-750; or 94-750 of SEQ ID NO: 2. In another preferred embodiment, the variant of PSA comprises the amino acid sequence set forth in SEQ ID NO: 4. Yet other preferred methods involve the destruction of other carcinoma cells. For example, melanoma cells can be destroyed using the method of the invention, wherein the target antigen is a MAGE or MART-1 antigen. Preferred variants of these antigens include the amino acid sequence set forth in SEQ ID NO: 10, 12, or 14. Pancreatic or colorectal cancer cells can be targeted by the use of a variant of carcino-embryonic antigen (CEA) including, e.g., the amino acid sequence set forth in SEQ ID NO: 6. Breast cancer cells can be targeted by the use of a variant of the target antigen is Her2/Neu including, e.g., the amino acid sequence set forth in SEQ ID NO: 8.

The polynucleotide encoding a variant of the target antigen may be included in a plasmid vector. For example, in one embodiment for treating prostate cancer in a subject, the subject will be administered a plasmid vector that contains an expression cassette encoding a variant of PSMA, e.g., comprising amino acids 44-750; 55-750; 61-750; 76-750; or 94-750 of SEQ ID NO: 2. In an even more preferred embodiment of the invention, the plasmid vector is termed "XC-PSMA-plasmid," having a Designation Number 203168, deposited on August 28, 1998 at the American Type Culture Collection, 10801 University Blvd., Manassas, Virginia 20110.

The polynucleotide encoding a variant of the target antigen may also be included in a viral vector, e.g., an adenoviral vector. For example, in one embodiment for treating prostate cancer in a subject, the subject will be administered a viral vector that is a replication-defective recombinant adenovirus comprising a nucleotide sequence encoding a variant of PSMA, e.g., comprising amino acids 44-750; 55-750; 61-750; 76-750; or 94-750 of SEQ ID NO: 2. In an even more preferred embodiment of the invention, the adenoviral vector is the viral vector termed "Ad5-PSMA," having ATCC Designation Number VR2361.

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In another embodiment of the invention, the undesired cells are cells that are reactive against an auto-antigen, i.e., a self-antigen, and the method is used, e.g., for treating auto-immune diseases. In another embodiment, the invention provides a method for preventing attack of a graft recipient by the graft, e.g., graft-versus-host disease, by selective destruction of cells reacting against the subject.

In yet another embodiment, the invention provides methods for treating diseases caused by, or associated with, an intracellular infectious agent, e.g., a virus. Diseases include malaria, colds, rabies, leishmaniasis, tuberculosis, and borrelia infection. According to the invention, cells presenting viral proteins can be targeted for destruction according to the invention by administering to the subject a polynucleotide encoding a variant of the foreign protein.

In another embodiment, the invention provides a method for treating a subject having undesired cells, comprising (i) obtaining professional antigen presenting cells from the subject; (ii) introducing into said cells *ex vivo* a polynucleotide encoding a polypeptide which is a variant of a target antigen of the undesired cells, which variant is not secreted from, nor expressed on the extracellular side of the cell membrane of, a cell in which it is expressed, and which variant is biologically inactive, to obtain cells that express the variant in association with MHC class I complexes; and (iii) administering the cells obtained in (ii) to the subject; such that the number of undesired cells is reduced in the subject

Also within the scope of the invention are plasmid vectors and viral vectors encoding a variant of a target antigen, e.g., a variant of the human PSMA, wherein the variant is not secreted from, nor expressed on the extracellular side of the cell membrane of, a cell in which it is expressed; and is preferably biologically inactive. Preferred plasmid and viral vectors comprise a nucleotide sequence encoding amino acids 44-750; 55-750; 61-750; 76-750; or 94-750 of SEQ ID NO: 2. In a preferred embodiment, the plasmid vector is "XC-PSMA" plasmid, which was deposited under the Budapest Treaty at the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, VA 20110-2209, on August 28, 1998, and assigned ATCC Deposit Number 203168. In another preferred embodiment, the viral vector is the adenoviral vector "Ad5 PSMA," which was deposited under the Budapest Treaty at the American Type

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Culture Collection (ATCC), 10801 University Boulevard, Manassas, VA 20110-2209, on August 28, 1998, and assigned ATCC Deposit Number VR 2362.

The present invention discloses, in particular, the construction of polynucleotides encoding variants of genetically modified forms of target antigens, e.g., tissue-specific or tumor antigens, and for the use of such constructs for immunotherapy of primary or metastatic cancer. The genetic modification of the antigens leads to expression of either functionally inactive products (variants) or prevents functionally active molecules from being secreted or expressed on the membrane of transfected cells. Such genetically modified antigens could also be fused to, or co-expressed with, other known immunogenic eukaryotic, prokaryotic or viral products or parts of them for the purpose of increasing the immunogenicity of the target antigens and bypassing the tolerance that may exist to them. Such genetic modifications may affect the antigenicity of the expressed protein, its primary structure or the generation of peptides available for binding to cell's MHC molecules, however, the variants must remain sufficiently immunogenic such as to induce a cellular immune response. The genetic modifications preferably will not significantly affect the efficiency of transcription and translation of the DNA, nor the translation of the RNA. Specifically the present invention discloses the development of a DNA, which leads to expression of a truncated form of the human prostate specific membrane antigen (PSMA). In particular, the examples describe a DNA construct with deletions of the membrane and the intracellular portions of the human PSMA. The resulting DNA encoding the extracellular portion of the PSMA (XC-PSMA) was incorporated in mammalian expression vectors. PSMA is a type II protein, it lacks a hydrophobic signal sequence and therefore is not secreted by the cell that produces it. Since the construct lacks membrane and cytoplasmic sequences, the resulting protein is not expressed on the membrane, therefore does not transduce signals and is not released from the membrane. Cells transfected with the XC-PSMA plasmid retain viability and express PSMA-derived peptides.

Furthermore, since the synthesized protein is not released but remains confined to the intracellular milieu, there is no production of antibodies directed against the protein and the immune response remains strictly cell-mediated. The exquisite engagement of cell-mediated immunity against particular antigen is very important especially in cases where the target antigen of interest is expressed on normal tissues that are anatomically

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sequestered in immuno-privileged sites such as the eye, brain, testis etc. Those tissues are inaccessible to cell mediated injury, but may readily be damaged by antibodies.

The present invention differs from the prior art at least in that it is believed to cause the dendritic cells to present an antigen derived from a target cell, e.g., a prostate cancer cell, on their surface through transfection with a polynucleotide of the invention, included in, e.g., a plasmid or adenovirus. As described herein, the transfection may occur *in vivo* using injected polynucleotide. Alternatively, the transfection may occur *in vitro* using purified DC precursor cells isolated from the prostate cancer patient's blood. If transfection is done *in-vitro*, the transfected cells are injected into the patient. Transfected DCs are superior to DCs, which have been primed with antigen *in vitro* because both their loading with antigen-derived peptide and their ability to stimulate killer cells are more efficient. In addition, *in vivo* transfection using, e.g., a plasmid or adenovirus, is less laborious and less expensive than *in vitro* methods. In addition, the use of transfected cells avoids the necessity of identifying peptides capable of binding to different HLA phenotypes, as is required in methods which involve the addition of peptides to cells. Finally, the use of a DNA sequence that encodes a variant of a target antigen, e.g., a truncated molecule of the PSMA, guarantees that the protein is not released by the transfected cells and no antibodies against the target protein that are potentially hazardous to normal brain tissue are produced. The methods of the present invention bypass the normal tolerance for self-antigens. This enables the cytolysis of target cells normally shielded from immune recognition.

### Brief Description of the Drawings

Fig.1 represents the amino acid sequence of human PSMA (SEQ ID NO: 2). The boxed region corresponds to the transmembrane domain.

Fig.2. Delayed type hypersensitivity (DTH) reaction in a prostate cancer patient at different time points following intradermal injection of either empty plasmid vector (circles), sGM-CSF (squares) or PSMA plasmid +sGM-CSF (triangles).

Fig.3. DNA immunization – Effect of CD86 and GM-CSF on immunization rate (development of DTH response 24 hours following third PSMA plasmid application)

1- Immunization with PSMA plasmid and CD86 plasmid (n=4)

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- 2- Immunization with PSMA plasmid and soluble GM-CSF (n=6)
- 3- Immunization with PSMA plasmid, CD86 plasmid and soluble GM-CSF (n=3)
- 4- Immunization with PSMA/CD86 combined plasmid and soluble GM-CSF (n=3)

Fig.4. PSA (diamonds) and DTH (squares) values for patient # 17, age 67. The patient had a 2 months doubling time of PSA before the onset of immunization. Immunizations were initiated at 7 months. His PSA values have been stable for almost one year. The patient is on immunotherapy only.

Fig.5. PSA (diamonds) and DTH (squares) values of patient # 5, age 58. Diagnosis about 6 months before "0 months" on the graph (T3cNoMo). Hormone therapy (Zoladex, Casodex) was initiated one month after diagnosis. Immunotherapy was initiated four months later (at "o" months in the graph). No tumor palpable at the time of prostatectomy (at 6 months on the graph). PSA became detectable at 9 months on the graph and it has been rising steadily despite immunotherapy.

Fig.6. PSA (diamonds) and DTH (squares) values of patient # 16, age 63. Diagnosis at one month (T2cNoMo). Several months after the 13 months date, no tumor but a small remnant of the prostate gland was palpable, no obstructive voiding symptoms.

Fig.7. PSA (diamonds) and DTH (squares) values of patient # 2.3, age 64. Diagnosis at one month with obstructive voiding symptoms, T2bNoMo. Biopsy Gleason pattern 3-4. Immunotherapy initiated at 7 x 14 days on the graph. Currently the patient has no obstructive voiding symptoms or any complains. The gland has shrunk significantly. Only a small nodule in the left lobe is still detectable on DRE and on ultrasonography.

Fig.8. PSA (diamonds) and DTH (squares) values of patient # 8, age 69. Diagnosed three years before the "0" month on the graph, and received TURP at that time. Tumor recurrence 3 months later. TURP three years after first TURP ("0" month on graph). Gleason pattern 3-4. Distant LN involvement. Hormone therapy (Zoladex, Casodex) started at 11 months. Immunotherapy initiated at 11 months. Hormone therapy discontinued at 16 months. Currently no obstructive voiding symptoms, no prostate palpable on DRE, no LN involvement on CAT scan.

Fig.9. Immunologic profile of prostate cancer patients prior to, and 15 months after, continuous IT.



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Fig. 10. Changes in selected flow cytometry parameters in responders and non-responders prior to, and 15 months after, continuous IT.

Fig. 11. Immunologic profile of patients with advanced colorectal cancer prior to and following chemotherapy, and of healthy volunteers.

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### Detailed Description of the Invention

The invention is based at least in part on the observation that administration of a nucleic acid encoding a variant of a prostate antigen, which variant is not secreted from, nor expressed on the extracellular side of the cell membrane of, the cell it is synthesized in, to subjects having advanced stages of prostate cancer was shown to improve the disease. As described in the Examples, dendritic cells transfected with a nucleic acid encoding the extracellular domain of PSMA are capable of activating CD8+ T cells *in vitro*, which are then capable of lysing target cells.

Accordingly, the present invention pertains generally to methods for eliminating undesired cells, e.g., in a subject. The undesired cells can be tumor cells, auto-reactive cells or cells infected with a foreign particle, such as a virally infected cell. It is well known that cell-mediated immune responses provide strong antiviral and anti-tumor mechanisms of protection. In a preferred embodiment, the invention provides methods for inducing a cell-mediated immune response against a target antigen, without inducing a significant humoral immune response against the target antigen. In a preferred embodiment, the invention comprises administering to a subject in need thereof, a nucleic acid encoding a variant of a target antigen, e.g., a portion of the antigen, which variant is not secreted from, or expressed on the extracellular side of the cellular membrane of, the cell it is synthesized in, which variant is preferably biologically inactive and/or prevents functionally active molecules from being secreted or expressed on the membrane of transfected cells. The target antigen can be homologous, e.g., a polypeptide from the subject, such as a tumor antigen, or heterologous, e.g., a viral polypeptide. In a preferred embodiment, the invention provides a method for treating a subject having tumor cells, comprising administering to the subject a pharmaceutically efficient amount of a nucleic acid encoding a variant of a tumor antigen, e.g., a portion of the extracellular domain of a tumor antigen that is normally expressed on the surface of tumor cells.

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Without wishing to be bound by a particular theory, it is believed that administration of a polynucleotide of the invention enters professional antigen presenting cells (APCs), where the polynucleotide is expressed into the variant of a target antigen, and then fragments of this variant antigen are presented on the surface of the APC together with MHC class I and class II complexes, resulting in activation of CD4+ and CD8+ T cells. Stimulation of CD4+ T cells seems to be essential because they provide the necessary signals for dendritic cell and CD8+ T cell maturation. CD8+ T cells will then lyse tumor cells expressing the target antigen. In view of the fact that the variant of the target peptide is not secreted or shed from the cells expressing it, no humoral immune response will be triggered. Furthermore, since the variant is biologically inactive, expression of the variant will not result in any undesirable effects in the subject.

In addition, without wishing to be held to this theory, it is also the inventors' belief that successful immunotherapy requires that the target antigen be presented by a DC simultaneously to both the helper (CD4+ T cells) and the effector (CD8+ T cells) arms of the immune system. Recognition by CD4+ T cells requires that antigenic peptides be expressed in conjunction with class II MHC molecules on the DC surface. This can be achieved by *in vivo* or *in vitro* transfection of DC with plasmid or infection of DC with recombinant adenovirus, which carry the DNA encoding the extracellular fragment of PSMA. In addition, transfection of dendritic cells with constructs encoding for known immunogenic eukaryotic, prokaryotic or viral products fused to, or co-expressed with, the variant of the target antigen (e.g., PSMA), increases the likelihood of breaking the tolerance towards said target antigen by stimulating "by-standing" CD4+ T cells reactive to said known immunogenic eukaryotic, prokaryotic or viral products.

#### Brief background on MHC class I antigen presentation

The study of CD8+T cell recognition of target cells has been extensive since the early 1970's when Zinkernagel and Doherty demonstrated that CTL recognition of viral-infected autologous target cells requires the presence of self class I MHC molecules. Thus such recognition of target cells by CD8+ T cells is referred to as being MHC class I-restricted. Zinkernagel, R. M., et al., Adv. Immunol. 27:51 (1979); Doherty, P. C., et al., Adv. Cancer Res. 42:1 (1984); and Zinkernagel, R. M., et al., Nature 248:701 (1974). It

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was later shown that virus-specificity of CTL's is directed against viral protein-derived peptide sequences that are presented by infected cell MHC class I molecules to CD8+T cells. See, for example, Townsend, A., et al., Cell 42:457 (1985) and Townsend, A., et al., Cell 44:959 (1986).

5           As noted above, it is not the entire antigen that is presented by target cells and recognized by CD8+ cells, but rather what is presented and recognized are small endogenously processed peptides that are generated from antigens by intracellular degradation pathways in either the cytosol or the endoplasmic reticulum ("ER") of the target cell. Such processed peptides bind to newly synthesized class I heavy chain- beta 2-  
10 microglobulin heterodimers in the ER. See, for example, Yewdell, J. W., et al., Science 244:1072 (1989); Townsend, A., et al., Cell 62:285 (1990); and Nuchtern, J. G., et al., Nature 339:223 (1989). The processed peptide is bound to the class I heavy chain-light chain dimer molecule via the class I antigen binding site/peptide cleft. The complex thereby generated is a transport competent trimer as reported by Yewdell, J. W., et al., Science  
15 244:1072 (1989); Townsend, A., et al., Cell 62:285 (1990); and Nuchtern, J. G., et al., Nature 339:223 (1989). This class I histocompatibility molecule-processed peptide complex is then expressed on the surface of the target cell where it may be ultimately recognized by T cell clonotypic receptors on CD8+ cells in conjunction with CD8 accessory molecules. See, Rotzschke, O., et al., Nature 348:252 (1990); Van Bleek, G. M., et al., Nature 348:213  
20 (1990); Rotzschke, O., et al., Science 249:283 (1990); and Falk, K., et al., Nature 348:248 (1990).

Although it was first believed that only MHC class I molecules could use the endogenous pathway of antigen presentation, it has now become clear that MHC class II molecules can also present antigen that has been synthesized in a cell. This is described,  
25 e.g., in Nuchtern et al. (1990) Nature 343: 74 and Loss et al. (1993) J. Exp. Med. 178: 73.

### Definitions

An "activated lymphocyte" is one that as a result of binding of a cognate antigen peptide:MHC molecule is producing polypeptide stimulatory factors (including, for  
30 example, cytokines) at a level that is elevated relative to the lymphocyte without the bound ligand.

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An "agretope" is the portion of an antigen or antigenic fragment which allows it to bind to an MHC molecule. A T cell epitope is an agretope.

"Antigen Presenting Cells", or "APC's" include known APC's such as Langerhans cells, veiled cells of afferent lymphatics, dendritic cells and interdigitating cells of lymphoid organs. The definition also includes mononuclear cells such as (1) lymphocytes and macrophages which take up and express polynucleotides according to the invention in skin and (2) mononuclear cells depicted on histological photographs contained herein. These cells are not tissue cells but are likely to be antigen presenting cells. The most important of these with respect to the present invention are those APC's which are known to be present in high numbers in epithelia and thymus dependent areas of the lymphoid tissues, including epidermis and the squamous mucosal epithelia of the buccal mucosa, vagina, cervix and esophagus (areas with "relatively high" concentrations of APC's). In addition to their definitions set forth below, therefore, "skin" and "mucosa" as used herein particularly refer to these sites of concentration of APC's. Further, "professional APCs" shall refer to cells whose primary purpose is antigen presentation; i.e., bone marrow derived dendritic cells.

"Antigen specific expression" refers to expression that occurs when the T cell recognizes its cognate antigen.

The term "biologically inactive" is used interchangeably herein with the term "functionally inactive," which, as applied to a polypeptide, refers to a polypeptide that has essentially no biological activity, i.e., no detectable biological activity that would be deleterious for its use within the context of the invention, and will not negatively affect the subject to which it is administered, in particular, e.g., it will not affect the cells taking up the nucleic acid and expressing the encoded polypeptide. Although a polypeptide may retain some negligible biological activity, it will be considered for the purposes herein, to be biologically inactive whenever the beneficial effect of the polypeptide significantly outweighs its negative effect resulting from residual biological activity. The term "biological activity" does not include antigenicity. Thus, a polypeptide that is biologically inactive is still capable of stimulating a humoral or cell-mediated immune response.

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The term "cellular immune response" is used interchangeably herein with "cell-mediated immune response" and refers to an immune response in which cells mediate the lysis of target cells.

"Cognate antigen" refers to an antigen, a peptide of which when associated with an MHC molecule forms a ligand that binds to a lymphocyte that recognizes it and causes triggering of signals for the effector function of the cell and/or for proliferation.

A CTL is "cytolytically specific for" cells expressing antigens if the CTL is capable of selectively recognizing and lysing the cells bearing the antigen. A CTL is "cytolytically reactive against" cells expressing antigens if the CTL is capable of lysing the cells bearing the antigen, without regard to its ability to selectively recognize such cells.

A "delivery complex" shall mean a targeting means (e.g. a molecule that results in higher affinity binding of a gene, protein, polypeptide or peptide to a target cell surface and/or increased cellular or nuclear uptake by a target cell). Examples of targeting means include: sterols (e.g. cholesterol), lipids (e.g. a cationic lipid, virosome or liposome), viruses (e.g. adenovirus, adeno-associated virus, and retrovirus) or target cell specific binding agents (e.g. ligands recognized by target cell specific receptors). Preferred complexes are sufficiently stable *in vivo* to prevent significant uncoupling prior to internalization by the target cell. However, the complex is cleavable under appropriate conditions within the cell so that the gene, protein, polypeptide or peptide is released in a functional form.

The term "endogenous" as used in the context of genes or other nucleic acids, or proteins present in a cell refers to a molecules which are naturally occurring in the cell, e.g., provided in the natural genome of the host organism or encoded thereby.

The term "exogenous" as used in the context of nucleic acids and proteins occurring in cells, refers to molecules which are not naturally present in the cell, and which have been, e.g., introduced by transfection or transduction of the cell (or the parent cell thereof).

As used herein, the term "gene" or "recombinant gene" refers to a nucleic acid molecule comprising an open reading frame and including at least one exon and (optionally) an intron sequence. The term "intron" refers to a DNA sequence present in a given gene which is not translated into protein and is generally found between exons.

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"Helper cells" or "T<sub>H</sub> cells" or "T helper cells" or "T helper lymphocytes" are a functional subclass of T cells which can help to generate cytotoxic T cells and cooperate with B cells in the production of an antibody response. Helper cells usually recognize antigen in association with class II MHC molecules.

5       The term "helper construct" refers generally to a nucleic acid molecule that includes nucleotide sequences providing functions deleted from a vector which is to be used to produce a transducing vector for delivery of a nucleotide sequence of interest. For example, AAV helper constructs are commonly used to provide transient expression of AAV rep and/or cap genes to complement missing AAV functions that are necessary for  
10   lytic AAV replication; however, helper constructs lack AAV ITRs and can neither replicate nor package themselves. AAV helper constructs can be in the form of a plasmid, phage, transposon, cosmid, virus, or virion. A number of AAV helper constructs have been described, such as the commonly used plasmids pAAV/Ad and pIM29 + 45 which encode both Rep and Cap expression products. See, e.g., Samulski et al. (1989) J. Virol. 63:3822-  
15   3828; and McCarty et al. (1991) J. Virol. 65:2936-2945. A number of other vectors have been described which encode Rep and/or Cap expression products. See, e.g., U.S. Pat. No. 5,139,941.

A "helper virus" is a virus which supplies some or all of the functions necessary for replication of a viral vector which are not encoded by a wild-type virus or which is encoded  
20   by a wild-type virus, but not by a recombinant virus. An "AAV helper virus" is a virus which supplies some or all of the functions necessary for AAV (and rAAV vector) replication which are not encoded by a wild-type AAV or which are encoded by a wild-type AAV, but not by a recombinant AAV. Typically these functions are supplied in trans by viruses such as adenovirus or herpes virus during viral replication. Thus, adenovirus and the  
25   herpes virus are examples of AAV helper viruses.

"Host" refers to the recipient of the therapy to be practiced according to the invention. The host may be any vertebrate, but will preferably be a mammal. If a mammal, the host will preferably be a human, but may also be a domestic livestock or pet animal.

30       The term "humoral immune response" refers to an immune response that involves antibodies.

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An "immune response" to a target antigen is the development in the host of a cellular and/or antibody-mediated immune response to the cell containing the target antigen (cellular immune reaction) or to soluble target antigen (antibody mediated response).

Usually, such a response comprises the individual producing CTLs and/or B cells and/or a  
5 variety of classes of T cells directed specifically to APCs expressing the target antigen.

"Infection," in the context of a viral-cell interaction refers to the process wherein a virus enters the cell in a manner whereby the genetic material of the virus can be expressed in the cell. A "productive infection" refers to the process wherein a virus enters the cell, is replicated, and then released from the cell.

10 "Infectious agent" refers to an agent which can infect cells, e.g., mammalian cells. An agent can be a microorganism, such as bacteria or a protozoan, a virus, or a prion.

"Intracellular infectious agent" refers to a microorganism or virus, in those cases where part or all of their replicative cycle occurs within the cells of an infected individual. Intracellular infectious agents include, for example, protozoa, fungi, bacteria, and viruses.

15 "Intracellular pathogen" refers to an agent capable of causing a disease state in a susceptible individual, in which part or all of its replicative cycle occurs within the cells of an infected individual. Intracellular pathogens include, for example, protozoa, fungi, bacteria, and viruses.

"Isolating the recombinant vector" refers to the process of purifying the  
20 encapsidated vector away from components found in the biological system (most typically tissue culture) from which it was produced. Procedures for the purification of biological materials, including vectors are well-known in the art, and may be found, e.g., in Sambrook and in Ausbel (both supra). Typically, viral particles (i.e., nucleic acids encapsidated in a capsid) are purified by CsCl gradient or step gradient centrifugation (see, Sambrook).

25 Typically, the vectors are approximately 90% pure after CsCl gradient centrifugation.

"Mucosa" refers to mucosal tissues of a host wherever they may be located in the body including, but not limited to, respiratory passages (including bronchial passages, lung epithelia and nasal epithelia), genital passages (including vaginal, penile and anal mucosa), urinary passages (e.g., urethra, bladder), the mouth, eyes and vocal cords.

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"Operably linked" refers to an arrangement of elements wherein the components so described are configured so as to perform their usual function. Thus, control sequences, e.g., transcriptional regulatory sequences, operably linked to a coding sequence are capable of effecting the expression of the coding sequence. The control sequences need not be  
5 contiguous with the coding sequence, so long as they function to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter sequence and the coding sequence and the promoter sequence can still be considered "operably linked" to the coding sequence.

"Naked polynucleotide(s)" refers to DNA or RNA or oligonucleotides. Naked in this  
10 context means polynucleotides, which are not complexed to colloidal materials (including liposomal preparations), or contained within a vector which would cause integration of the polynucleotide into the host genome.

The "non-human animals" of the invention include mammals such as rodents, non-human primates, sheep, dog, cow, chickens, amphibians, reptiles, etc. Preferred non-  
15 human animals are selected from the rodent family including rat and mouse, most preferably mouse, though amphibians, such as members of the *Xenopus* genus, and chickens are also included.

As used herein, the term "nucleic acid" refers to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term  
20 should also be understood to include, as equivalents, analogs of either RNA or DNA made from nucleotide analogs, and, as applicable to the embodiment being described, single (sense or antisense) and double-stranded polynucleotides.

A "packaging cell" refers to a host cell which, by way of stable or transient transfection with heterologous nucleotide sequences, harbors a nucleic acid molecule  
25 comprising a viral helper construct, wherein the construct is capable of providing transient expression of viral helper functions that can be provided in trans for productive viral replication. Expression of the viral helper functions can be either constitutive, or inducible, such as when the helper functions are under the control of an inducible promoter. An example of a packaging cell is a 293 cell.



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A "packaging signal" refers to a nucleotide sequence, which when present in a nucleic acid allows the nucleic acid to be packaged into a viral particle in appropriate conditions, such as in the presence of packaging extracts or in a packaging cell.

5 "Polynucleotide of the invention" refers to a polynucleotide encoding a variant of a target antigen, which variant is not secreted from, or expressed on the extracellular side of the cell membrane of, the cell in which it is synthesized; and which is biologically inactive.

The term "polypeptide" refers to a polymer of amino acids and does not refer to a specific length of the product; thus, peptides, oligopeptides, and proteins are included within the definition of polypeptide. This term also does not refer to or exclude post-  
10 expression modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like. Included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.), polypeptides with substituted linkages, as well as other modifications known in the art, both naturally occurring and non-naturally occurring.

15 "Polypeptide of the invention" refers to a polypeptide which is encoded by a polynucleotide of the invention.

A "producer cell" refers to a packaging cell that has been stably or transiently transfected with a viral vector-either before, subsequent to, or at the same time as transfection of the cell with the viral helper functions. In this manner, a producer cell  
20 contains viral sequences that are provided in cis for replication and packaging (e.g., functional ITR sequences), and viral sequences encoding helper functions missing from the viral vector and provided in trans for replication and packaging. In the presence of requisite viral helper functions, the producer cell is thus capable of encoding viral polypeptides that are required for packaging transfected viral DNA (e.g., AAV viral vectors containing a  
25 recombinant nucleotide sequence of interest) into infectious viral particles for subsequent gene delivery.

As used herein, the term "promoter" means a DNA sequence that regulates expression of a selected DNA sequence operably linked to the promoter, and which effects expression of the selected DNA sequence in cells. The term encompasses "tissue specific"  
30 promoters, i.e. promoters, which effect expression of the selected DNA sequence only in specific cells (e.g. cells of a specific tissue). The term also covers so-called "leaky"

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promoters, which regulate expression of a selected DNA primarily in one tissue, but cause expression in other tissues as well. The term also encompasses non-tissue specific promoters and promoters that constitutively express or that are inducible (i.e. expression levels can be controlled).

5           The term "recombinant" when used with reference to a cell or virus indicates that the cell or virus contains a nucleic acid whose origin is exogenous to the cell or virus type. Thus, for example, recombinant cells express genes that are not found within the native (non-recombinant) form of the cell.

10           A "recombinant expression cassette" is a nucleic acid construct, generated recombinantly or synthetically, with a series of specified nucleic acid elements which permit transcription of a particular nucleic acid. The recombinant expression cassette can be part of a plasmid, virus, or nucleic acid fragment. Typically, the recombinant expression cassette includes a nucleic acid to be transcribed, and a promoter. In some embodiments, the expression cassette also includes, e.g., an origin of replication, and/or chromosome  
15 integration elements.

          The term "recombinant expression vector" refers to a replicable unit of DNA or RNA in a form capable of being introduced into a target cell by transformation, electroporation, transduction or viral infection, and which codes for the expression of a heterologous structural coding sequence, for example, a cytokine, which is transcribed into  
20 mRNA and translated into protein under the control of elements having a regulatory role in gene expression. Such vectors will preferably also contain appropriate transcription and translation control sequences, including initiation sequences operably linked to the coding sequence.

          A "recombinant helper nucleic acid" or more simply helper nucleic acid is a nucleic  
25 acid which encodes functions which allow a nucleic acid to be encapsidated in a capsid. Typically, in the context of the present invention, the helper plasmid, or other nucleic acid, encodes viral functions and structural proteins which allow a recombinant viral vector to be encapsidated into a capsid. In one preferred embodiment, a recombinant AAV helper nucleic acid is a plasmid encoding AAV polypeptides, and lacking the AAV ITR regions.  
30 For example, in one embodiment, the helper plasmid encodes the AAV genome, with the exception of the AAV ITR regions, which are replaced with adenovirus ITR sequences.

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This permits replication and encapsidation of the AAV replication defective recombinant vector, while preventing generation of wild-type AAV virus, e.g., by recombination.

The term "recombinant protein" refers to a polypeptide, which is produced by recombinant DNA techniques. Thus a protein that is encoded by a nucleic acid which is introduced into a cell, as opposed to being expressed from the naturally-occurring genomic DNA of the cell, is a recombinant protein. Moreover, the phrase "a first polypeptide derived from a second polypeptide", refers to a first polypeptide that is identical to the second polypeptide but for the introduction of one or more amino acid changes therein.

A "recombinant virion," such as "rAAV", is defined herein as an infectious, replication-defective virus composed of a protein shell, encapsidating a viral vector comprising a heterologous nucleotide sequence of interest.

"Sequences necessary for viral packaging" in the context of a viral helper nucleic acid include viral sequences active in trans which encode proteins necessary for encapsidation of the viral vector into an infectious particle. For instance, the packaging proteins for AAV can include capsid proteins (Vp1, Vp2, Vp3) and replicase proteins (Rep 78, Rep 68, Rep 40, Rep 52).

The term "signal peptide" is used interchangeably herein with "signal sequence" and "leading sequence" to refer to a sequence or portion of a polypeptide that is located at the N-terminus of a polypeptide and which associates with the endoplasmic reticulum and mediates the secretion or membrane attachment of a polypeptide.

"Skin" as used herein refers to the epidermal, dermal and subcutaneous tissues of a host.

As used herein, the term "specifically hybridizes" or "specifically detects" refers to the ability of a nucleic acid molecule of the invention to hybridize to at least approximately 6, 12, 20, 30, 50, 100, 150, 200, 300, 350, 400 or 425 consecutive nucleotides of a vertebrate.

The term "target antigen" used interchangeably herein with "target peptide" refers to an antigen, which is presented on the surface of a target cell in association with an MHC class I complex. The target antigen can be a polypeptide, or a derivative thereof, such as a glycoprotein.

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A "target cell" refers to a cell expressing a target antigen, which is targeted for lysis by a cell-mediated immune reaction.

A "target tissue" refers to a tissue containing target cells.

5 A "Tc" or "cytolytic T cell" or "CTL" or "cytotoxic T cell" refers to a CD8+ T cells which is capable of lysing a cell.

"TH1 Response(s)" refers to a cellular immune response that is induced preferentially by antigens that bind to and activate certain APC's; i.e., macrophages and dendritic cells.

10 A "therapeutically efficient amount" or "pharmaceutically efficient amount" of a compound, e.g., polynucleotide of the invention, refers to a dose sufficient to induce an immune response against the target antigen.

15 "Titers" are numerical measures of the "concentration" of a virus or viral vector compared to a reference sample, where the concentration is determined either by the activity of the virus, or by measuring the number of viruses in a unit volume of buffer. The titer of viral stocks are determined, e.g., by measuring the infectivity of a solution or solutions (typically serial dilutions) of the viruses, e.g., on HeLa cells using the soft agar method (see, Graham & Van Der eb (1973) Virology 52:456-467) or by monitoring resistance conferred to cells, e.g., G418 resistance encoded by the virus or vector, or by quantitating the viruses by UV spectrophotometry (see, Chardonnet & Dales (1970) Virology 40:462-477).

As used herein, the term "transfection" means the introduction of a nucleic acid, e.g., via an expression vector, into a recipient cell by nucleic acid-mediated gene transfer. "Transformation", as used herein, refers to a process in which a cell's genotype is changed as a result of the cellular uptake of exogenous DNA or RNA.

25 "Transcriptional regulatory sequence" is a generic term used throughout the specification to refer to DNA sequences, such as initiation signals, enhancers, and promoters, which induce or control transcription of protein coding sequences with which they are operably linked.

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The term "treating" as used herein is intended to encompass curing as well as ameliorating at least one symptom of the condition or disease and includes prophylactic and therapeutic methods.

5 "Tumor antigen" or "tumor-associated antigen" refers to an antigen that is found on tumor cells, and which may be specific to certain tumors and/or specific to tumor cells relative to normal cells. A tumor antigen can also be an embryonic protein that has been re-expressed by transformed cells or an autoantigen that is not truly tumor specific, but is present in mammalian tumor tissue.

10 A "variant" of a target antigen refers to all or a portion of a target antigen or modified form thereof, that (i) is sufficiently homologous to the target antigen to ensure that CD8+ T cells recognizing the variant will also recognize the target antigen; and (ii) is not secreted from, or expressed on the extracellular side of the cell membrane of, the cell in which it is expressed. A preferred variant is biologically (i.e., functionally) inactive.

15 The term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of preferred vector is a form of vector that can be maintained episomally, i.e., a nucleic acid capable of extra-chromosomal replication.

20 Preferred vectors are those capable of autonomous replication and/or expression of nucleic acids to which they are linked. Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of "plasmids" which refer generally to circular double stranded DNA loops which, in their vector form are not bound to the chromosome. In the present specification, "plasmid" and "vector" are used interchangeably as the plasmid is the most commonly used form of  
25 vector.

A "viral vector" refers to a nucleic acid containing at least a portion of a viral genome sufficient for replication and packaging in the presence of an appropriate helper virus and appropriate cell line or packaging extract. For example, by an "AAV vector" is meant a vector derived from an adeno-associated virus serotype, including without  
30 limitation, AAV-1, AAV-2, AAV-3, AAV-4, AAV-5, AAVX7, etc. AAV vectors can have one or more of the AAV wild-type genes deleted in whole or part, preferably the rep and/or

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cap genes, but retain functional flanking ITR sequences. Functional ITR sequences are necessary for the rescue, replication and packaging of the AAV virion. Thus, an AAV vector is defined herein to include at least those sequences required in cis for replication and packaging (e.g., functional ITRs) of the virus. The ITRs need not be the wild-type  
5 nucleotide sequences, and may be altered, e.g., by the insertion, deletion or substitution of nucleotides, so long as the sequences provide for functional rescue, replication and packaging.

By "virion" or "viral particle" is meant a complete virus particle, such as a wild-type (wt) virus particle (comprising a nucleic acid genome associated with a capsid protein coat),  
10 or a recombinant virus particle as described below. For example, by "adenoviral virion" is meant a complete virus particle, such as a wild-type (wt) Ad virus particle comprising an Ad nucleic acid genome associated with an Ad capsid protein coat, or a recombinant AAV virus particle as described below. In this regard, single-stranded AAV nucleic acid molecules of either complementary sense, e.g., "sense" or "antisense" strands, can be  
15 packaged into any one AAV virion and both strands are equally infectious.

#### Target antigens and target diseases and conditions

The methods of the invention permit the destruction of any undesirable cell, such as a cell of a subject, which has lost some of its normal characteristics or a cell infected with a  
20 foreign particle, e.g., a microorganism. Accordingly, the invention provides methods for reducing or eliminating uncontrolled cell proliferation, e.g., of malignant cells. The invention provides immunotherapy methods for primary and metastatic cancer.

Undesirable cells also include those, which produce an undesirable protein or other molecule. For example, autoimmune diseases can be treated by using the method of the  
25 invention against a cell producing auto-antibodies or against a lymphocyte that is auto-reactive, i.e., which recognizes an endogenous antigen. Similarly, allergic reactions, transplant rejections, and other immunological disorders can be treated by destruction of the cell causing the disease. Yet other diseases and conditions that can be treated according to the method of the invention include those, which are associated with, e.g., caused by,  
30 infectious agents, e.g., viruses, bacteria, protozoans, and prions, which infectious agent is an intracellular infectious agent, which can be killed by lysis of the cell containing it.

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Even normal cells of a subject which are found to be undesirable, may be eliminated by the method of the invention. In one embodiment, such undesirable normal cells are first modified by expressing in the cells an antigen which will be used as the target antigen for the method of the invention. Thus, generally, any undesirable cell may be "tagged" for  
5 destruction.

In a preferred embodiment of the invention, a polynucleotide encoding a variant of a target antigen, which is expressed in the undesirable cell, is administered to the subject in need of elimination of the undesirable cells. It is preferred that the target antigen against which a cellular immune reaction will be mounted is expressed preferentially in the  
10 undesirable cells, and/or that the undesirable cells are preferentially reached by the cytolytic cell, such as not to unnecessarily destroy non-target cells, such as normal cells. Target antigens may, however, be expressed on normal tissues that are anatomically sequestered in immuno-privileged sites such as the eye, brain, testis etc., since these tissues are inaccessible to cell mediated injury, and only damaged by antibodies. Since the method of  
15 the invention induces a cellular immune response without inducing a significant humoral immune response, thus without the production of antibodies, immuno-privileged tissues expressing the target antigen will be protected from lysis. For example, immunotherapy based on eliciting cellular responses to differentiation (tyrosinase; gp100; TRP1; TRP2; MART-I/Melan-A; membrane-associated mucin, MUC- I mucin) or normal tissue-specific  
20 (PSMA, PSA) antigens constitute an example where the production of antibodies against the target is undesirable.

The target antigen chosen for inducing the destruction of the undesirable cells is preferably an antigen containing a least one immunogenic peptide or T cell epitope, i.e., a peptide that can be presented to CD8+ T cells within the context of MHC class I  
25 complexes. Preferably the target antigen comprises at least 2, 3, 5, 10 or more T cell epitopes, since this will allow the preparation of a variant of the target antigen to comprise more than one T cell epitope, thereby increasing the likelihood of a strong immune reaction. Since peptides associate with MHC class I complexes in the endoplasmic reticulum (ER), the target peptide may be preferably a peptide of which at least a portion will be targeted to  
30 the ER, and then associate with an MHC class I complex. Thus, preferred target antigens include any naturally-occurring secreted or membrane protein, i.e., a protein that migrates

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through the ER during its synthesis. Yet other preferred antigens include foreign or heterologous proteins, e.g., proteins from microorganisms. It has been shown that some foreign proteins, such as viral proteins (e.g., influenza nucleoprotein), which are neither membrane bound nor secreted, i.e., they do not gain access to exocytic pathways in their intact form, are presented on the cell surface in association with MHC class I. Thus, generally, the target antigen does not have to be secreted or a membrane protein, but it must merely contain at least a portion, which is capable of associating with an MHC class I complex of the target cell, and be able to be recognized within the context of MHC class I by CD8<sup>+</sup> T cells.

The target antigen can thus be a eukaryotic protein, a prokaryotic protein or a viral protein. A target peptide can be derived from a cytosolic protein, a secreted protein, a transmembrane protein or a nuclear protein.

Preferred eukaryotic proteins include tumor antigens. Malignant tumors express a number of proteins that can serve as target antigens for an immune attack. These molecules include but are not limited to tissue-specific antigens such as MART-1, tyrosinase and GP 100 in melanoma and prostatic acid phosphatase (PAP) and prostate-specific antigen (PSA) in prostate cancer. Other target molecules belong to the group of transformation-related molecules such as the oncogene HER-2/Neu/ErbB-2. Yet another group of target antigens are onco-fetal antigens such as carcinoembryonic antigen (CEA). In B-cell lymphoma the tumor-specific idiotype immunoglobulin constitutes a truly tumor-specific immunoglobulin antigen that is unique to the individual tumor. B-cell differentiation antigens such as CD19, CD20 and CD37 are other candidates for target antigens in B-cell lymphoma. Some of these antigens (CEA, HER-2, CD 19, CD20, idiotype) have been used as targets for passive immunotherapy with monoclonal antibodies with limited success. Thus, the antigen can be a product of a completely silent gene (e.g., MAGE or RAGE (Gaugler et al. (1996) Immunogenetics 44:323)), a differentiation antigen (e.g., CEA and gp75 (Wang et al. (1996) J. Exp. Med. 183:1131), antigens resulting from mutations (e.g., bcr-abl in myeloid leukemias; Bosch et al. (1996) Blood 88:3522); high-density normal antigens (e.g., Her2/Neu, p53 and PRAME (Ikeda et al. (1997) Immunity 6:99); or a tissue specific antigen.



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A preferred tumor antigen is prostate specific membrane antigen (PSMA) or an alternatively spliced form thereof, which are described in U.S. patent Nos. 5,538,866 and 5,935,818 by Israeli et al. PSMA expression is an integral transmembrane protein which is restricted to prostate epithelial cells (Horoszewicz JS, Kawinski E and Murphy GP.

5 Monoclonal antibodies to a new antigenic marker in epithelial prostate cells and serum of prostate cancer patients. *Anticancer Res.* 7:927;1987) and human brain tissue (Luthi Carter R, Barczak AK, Speno H, Coyle JT. Molecular characterization of human brain N acetylated alpha-linked acidic dipeptidase (NAALADase). *J Pharmacol. Exp. Therap.* 286:1020;1998). The antigen is expressed on normal and neoplastic prostate cells in the  
10 prostate and in prostate tumor metastases. Thus, prostate cancer can be treated, e.g., by administering to a subject having prostate cancer a polynucleotide encoding a portion of PSMA which, when expressed in cells, is not secreted, nor expressed on the extracellular side of the cellular membrane, and which is essentially biologically inactive, e.g., at least a portion of the extracellular domain.

15 In an exemplary embodiment, treatment of prostate cancer, dendritic cells (DCs) of the patients are prepared by transfection using, e.g., a plasmid or a recombinant replication-deficient adenovirus whose DNA includes DNA encoding, e.g., a truncated fragment of the prostate specific membrane antigen. Dendritic cells may be transfected *in vivo* by injection of the plasmid or recombinant replication-deficient adenovirus in the patient. Alternatively  
20 the DC may be transfected (infected) *in vitro* by treating isolated dendritic cell precursor cells with the plasmid or recombinant replication-deficient adenovirus. The dendritic cells are then injected into the patient.

In one method of treating of prostate cancer patients, the plasmid is injected intradermally, sub-cutaneously, intramuscularly, intrathecally or through the blood  
25 circulation. In another method of treatment, the plasmid is first incorporated into the genome of a replication- deficient adenovirus which is injected intradermally, sub-cutaneously, intramuscularly, intrathecally or through the blood circulation. into a patient. In yet another method of treatment, CD 14+ monocyte cells of a prostate cancer patient are isolated and matured into dendritic cells and transfected with either the plasmid or the  
30 adenovirus of the first two methods. The DCs are then stimulated to express MHCs and are

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infused back into the prostate cancer patient where they stimulate autologous T-cells. These stimulated T-cells then destroy malignant and some normal prostate cells.

One effect of all of these treatments is to either by-pass the normal tolerance for self-antigens or the tolerance to tumor antigens. This will enable the cytolysis of target malignant prostate cells normally shielded from immune recognition. The destruction of normal prostate cells in the process is not detrimental to the patient because a malignant prostate (with its mixture of normal and malignant cells) customarily is destroyed through surgery or radiation in the primary treatment for this disease. However, normal brain cells, which also express PSMA, will not be destroyed, since CTLs do not access the brain and, since no antibodies (which can access the brain) are produced according to the method of the invention.

Other prostate specific antigens include the prostatic acid phosphatase (PAP). Elevated levels of PAP in the bloodstream are considered indicative of prostate cancer, and this enzyme has been widely studied (Yam, *Amer J Med* (1974) 56:604. Improved methods of cancer detection using this enzyme were described by Chu et al. in PCT application WO79/00475. The structure of the enzyme has also been studied by Sharief, F. S., et al., *Biochem Biophys Res Commun* (1992) 184:1468-1476 and by Van Etten, R. L., et al., *J Biol Chem* (1991) 266:9993-9999. The nucleotide sequence encoding human PAP has been determined from a full length cDNA clone (Sharief, F. S., et al., *Biochem Biophys Res Commun* (1989) 180:79-86; Tailor, P. G., et al., *Nucleic Acids Res* (1990) 18:4928.

In addition to PAP, other suitable candidates for antigens over-represented on prostate tissue are known. Most prominent among these is "prostate specific antigen" or "PSA". PSA is present in the epithelial cells comprising the prostatic ductal elements. It has been demonstrated in all primary and metastatic prostatic tumors tested and in normal benign prostate but not in non-prostatic cancer tissues or in normal tissues other than prostate. The complete amino acid sequence of PSA from human seminal plasma has been determined (Watt KW et al., *Proc Natl Acad Sci USA* (1986) 83:3166-3170). PSA consists of a single polypeptide chain with 240 amino acid residues and has a calculated molecular weight of 26,496. Carbohydrate side chains are possibly attached. The cDNA encoding PSA has been produced and characterized (Lundwall A, Lilja, H, *FEBS Lett* (1987) 214:317-322; Schultz P, et al., *Nucleic Acids Res* (1988) 16:6226; and Henttu P and Bihko

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P, Biochem and Biophys Res Commun (1989) 160:903-910). The gene for the PSA has also been characterized (Lundwall A, Biochem and Biophys Res Commun (1989) 162:1151-1159, Riegman, PHJ, et al., Biochem and Biophys Res Commun (1989) 159:103-111 and Klobeck G, et al., Nucleic Acids Res 1989 17:3981.)

5           In another illustrative embodiment, the target antigen is the Her-2/neu antigen, which is a member of the epidermal factor receptor family and is presumed to function as a growth receptor (Beckmann et al., 1992, Eur. J. Cancer 28:322). It is a transmembrane protein and is expressed during fetal development and, very weakly on normal cells (as a single copy). Amplification of the gene and/or overexpression of the associated protein  
10   have been identified in many human cancers such as breast, gynecological, ovary, uterus, stomach, prostate or lung. Accordingly, in one embodiment, DNA encoding a truncated form of the Her 2/neu protein lacking the transmembrane portion and the leading sequence can be constructed and included in a plasmid or viral vector(s), and administered to a subject having breast, ovary, uterine, prostate or lung cancer.

15           Another antigen that can be targeted is the melanocyte differentiation antigen MART-1, which is a common melanoma antigen recognized by many CTLs from melanoma patients (Coulie et al., 1994, J. Exp. Med. 180:35; Hawakami et al., 1994, PNAS 91:3515; Bakker et al., 1994, J. Exp. Med. 179: 1005). MART-1 (also referred to as MART-1/Melan A) is a membrane protein of 118 amino acids having a single  
20   transmembrane domain. It was recently shown that dendritic cells transduced with a retroviral vector expressing a melanoma tumor-associated antigen gene (MART-1) properly presented the MART-1 antigen, and that the resulting dendritic cells raised a strong CTL mediated anti-melanoma tumor response *in vivo* (see, Reeves, et al. (1996) Cancer Research 56:5672-5677). Thus, DNA encoding a truncated form of this antigen with no  
25   transmembrane domain or, a full-size protein with no leading sequence, can be included in a plasmid or viral expression vector and used for immunotherapy of melanoma patients.

          Other melanoma associated antigens include gp100, tyrosinase/albino, p97 melanoma antigen, and any of the various MAGEs (melanoma associated antigen E), including MAGE 1, 2, 3, 4, etc. (Boon, T. Scientific American (March 1993):82-89; e.g.,  
30   Zhao, et al., J. Immunol., 156(2):700-10 (1996); Kawakami, et al., J. Exp. Med.,

180(1):347-52 (1994); and Topalian, et al., Proc. Natl. Acad. Sci. USA, 91(20):9461-5 (1994)).

Proteins associated with breast cancer that can be used as target antigens, include bcl-1, bcl-2, vasopressin related proteins; see, North, et al., Breast Cancer Res. Treat., 5 34(3):229-35 (1995); Hellemans, Br. J. Cancer, 72(2):354-60 (1995); and Hurlimann, et al., Virchows Arch., 426(2). 163-8 (1995)). Antigens associated with other carcinomas include, e.g., c-myc, int-2, hst-1, ras and p53 mutants; see, Issing, et al., Anticancer Res., 13(6B):2541-51 (1993); Tjoa, et al., Prostate, 28(1):65-9 (1996); Suzich, et al., Proc. Natl. Acad. Sci. USA, 92(25):11553-7 (1995); and Gjertsen, et al., Lancet, 346(8987):1399-400 10 (1995)). B cell lymphoma tumor antigens that can be used include CD19, CD20, CD37, as well as an antigen described in U.S. 6,099,846. An antigen associated with renal carcinoma is RAGE (Gaugler et al. (1996) Immunogenetics 44:323).

For treating cancer of the pancreas or colorectal cancer, a variant derived from carcinoembryonic antigen (CEA) can be used. Carcinoembryonic antigen is localized to 15 epithelia of the intestinal lumen (see, e.g., Benchimol, et al., Cell, 57:327-324, 1989).

Thus, generally, the invention can be used to treat numerous types of cancers, including solid tumors as well as cancers of blood cells, lymphomas and leukemias. Numerous antigens associated with any number of cancers, which can be used according to the invention, including ovarian, breast, colorectal, melanoma, pancreas, stomach, gall 20 bladder, oesophagus, lung, gliomas, renal, thyroid, are set forth in US 6,093,399.

Target antigens can be from viruses, e.g., viruses that result in chronic infections, for example, the hepadnaviruses (including HBV), the lentiviruses (including HIV), herpesviruses (including HSV-1, HSV-2, EBV, CMV, VZV, and HHV-6), and the flaviviruses/pestiviruses (including HCV). Also included, as viruses that cause chronic 25 viral infections are human retroviruses, for example, human T lymphotropic viruses (HTLV-1 and HTLV-2) that cause T cell leukemia and myelopathies. Other organisms that cause chronic infections include, for example, pathogenic protozoa, (e.g., Pneumocystis carinii, trypanosoma, malaria and Toxoplasma gondii), bacteria (e.g., mycobacteria, salmonella and listeria) and fungi (e.g. candida and aspergillus).

30 The nucleotide sequences of a number of these viruses, including different species, strains, and isolates are known in the art. For reviews see: Robinson (1990)

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(Hepadnaviridae); Levy, Microbiological Reviews, 57:183-289 (1993) (HIV); and Choo et al., Seminars in Liver Disease, 12:279-288 (1992) (HCV). Particularly suitable target antigens are those which induce a T cell response, and particularly a CTL-response during infection. These may include, for example, from HBV, the core antigen, the E antigen, and the surface antigen (HBsAg). Polynucleotide sequences for HBsAg including the pre-S1, pre-S2 and S regions from a variety of surface antigen subtypes are known in the art. See, for example, Okamoto et al., J. Gen. Virol., 67:1383-1389 (1986); Genbank accession numbers D00329 and D00330. The polynucleotide sequences encoding HIV glycoprotein gp160 and other antigenic HIV regions are known in the art. Lautenberger et al., Nature, 313:277-284 (1985); Starcich et al., Cell, 45:637-648 (1986); Wiley et al., Proc. Natl. Acad. Sci. USA, 83:5038-5042 (1986); and Modrow et al., J. Virol., 61:570-578 (1987).

In particular, the present invention will find use for stimulating an immune response against a wide variety of proteins from the herpesvirus family, including proteins derived from herpes simplex virus (HSV) types 1 and 2, such as HSV-1 and HSV-2 glycoproteins gB, gD and gH; antigens derived from varicella zoster virus (VZV), Epstein-Barr virus (EBV) and cytomegalovirus (CMV) including CMV gB and gH; and antigens derived from other human herpesviruses such as HHV6 and HHV7. (See, e.g. Chee et al., Cytomegaloviruses (J. K. McDougall, ed., Springer-Verlag 1990) pp. 125-169, for a review of the protein coding content of cytomegalovirus; McGeoch et al., J. Gen. Virol. (1988) 69:1531-1574, for a discussion of the various HSV-1 encoded proteins; U.S. Pat. No. 5,171,568 for a discussion of HSV-1 and HSV-2 gB and gD proteins and the genes encoding therefor; Baer et al., Nature (1984) 310:207-211, for the identification of protein coding sequences in an EBV genome; and Davison and Scott, J. Gen. Virol. (1986) 67:1759-1816, for a review of VZV.)

Antigens from the hepatitis family of viruses, including hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), the delta hepatitis virus (HDV), hepatitis E virus (HEV) and hepatitis G virus (HGV), can also be conveniently used in the techniques described herein. By way of example, the viral genomic sequence of HCV is known, as are methods for obtaining the sequence. See, e.g., International Publication Nos. WO 89/04669; WO 90/11089; and WO 90/14436. The HCV genome encodes several viral proteins, including E1 (also known as E) and E2 (also known as E2/NSI) and an N-terminal

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nucleocapsid protein (termed "core") (see, Houghton et al., *Hepatology* (1991) 14:381-388, for a discussion of HCV proteins, including E1 and E2). Each of these proteins, as well as antigenic fragments thereof, will find use in the present methods.

Similarly, the sequence for the delta -antigen from HDV is known (see, e.g., U.S. Pat. No. 5,378,814) and this antigen can also be conveniently used in the present methods. Additionally, antigens derived from HBV, such as the core antigen, the surface antigen, sAg, as well as the presurface sequences, pre-S1 and pre-S2 (formerly called pre-S), as well as combinations of the above, such as sAg/pre-S1, sAg/pre-S2, sAg/pre-S1/pre-S2, and pre-S1/pre-S2, will find use herein. See, e.g., "HBV Vaccines-from the laboratory to license: a case study" in Mackett, M. and Williamson, J. D., *Human Vaccines and Vaccination*, pp. 159-176, for a discussion of HBV structure; and U.S. Pat. Nos. 4,722,840; 5,098,704; 5,324,513; Beames et al., *J. Virol.* (1995) 69:6833-6838, Birnbaum et al., *J. Virol.* (1990) 64:3319-3330; and Zhou et al., *J. Virol.* (1991) 65:5457-5464.

Antigens derived from other viruses will also find use in the claimed methods, such as without limitation, proteins from members of the families Picornaviridae (e.g., polioviruses, etc.); Caliciviridae; Togaviridae (e.g., rubella virus, dengue virus, etc.); Flaviviridae; Coronaviridae; Reoviridae; Birnaviridae; Rhabdoviridae (e.g., rabies virus, etc.); Filoviridae; Paramyxoviridae (e.g., mumps virus, measles virus, respiratory syncytial virus, etc.); Orthomyxoviridae (e.g., influenza virus types A, B and C, etc.); Bunyaviridae; Arenaviridae; Retroviridae (e.g., HTLV-I; HTLV-II; HIV-1 (also known as HTLV-III, LAV, ARV, hTLR, etc.)), including but not limited to antigens from the isolates HIV[IIIb], HIV[SF2], HIV[LAV], HIV[LAI], HIV[MN]); HIV-1[CM235], HIV-1[US4]; HIV-2; simian immunodeficiency virus (SIV) among others. Additionally, antigens may also be derived from human papillomavirus (HPV) and the tick-borne encephalitis viruses. See, e.g. *Virology*, 3rd Edition (W. K. Joklik ed. 1988); *Fundamental Virology*, 2nd Edition (B. N. Fields and D. M. Knipe, eds. 1991), for a description of these and other viruses.

More particularly, the gp120 envelope proteins from any of the above HIV isolates, including members of the various genetic subtypes of HIV, are known and reported (see, e.g., Myers et al., *Los Alamos Database*, Los Alamos National Laboratory, Los Alamos, N.M. (1992); Myers et al., *Human Retroviruses and Aids*, 1990, Los Alamos, N.M.: Los Alamos National Laboratory; and Modrow et al., *J. Virol.* (1987) 61:570-578, for a

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comparison of the envelope sequences of a variety of HIV isolates) and antigens derived from any of these isolates will find use in the present methods. Furthermore, the invention is equally applicable to other immunogenic proteins derived from any of the various HIV isolates, including any of the various envelope proteins such as gp160 and gp41, gag  
5 antigens such as p24gag and p55gag, as well as proteins derived from the pol region.

Influenza virus is another example of a virus for which the present invention will be particularly useful. Specifically, the envelope glycoproteins HA and NA of influenza A are of particular interest for generating an immune response. Numerous HA subtypes of influenza A have been identified (Kawaoka et al., *Virology* (1990) 179:759-767; Webster et  
10 al., "Antigenic variation among type A influenza viruses," p. 127-168. In: P. Palese and D. W. Kingsbury (ed.), *Genetics of influenza viruses*. Springer-Verlag, New York). Thus, proteins derived from any of these isolates can also be used in the immunization techniques described herein.

The methods described herein will also find use with numerous bacterial antigens,  
15 such as those derived from organisms that cause diphtheria, cholera, tuberculosis, tetanus, pertussis, meningitis, and other pathogenic states, including, without limitation, *Meningococcus* A, B and C, *Hemophilus influenza* type B (HIB), and *Helicobacter pylori*. Examples of parasitic antigens include those derived from organisms causing malaria and Lyme disease.

20 Target antigens that can be used according to the method of the invention are also described in the following documents: U.S. Pat. No. 5,338,683 (e.g., DNA encoding Herpesvirus glycoproteins, inter alia); U.S. Pat. Nos. 5,494,807, 5,756,103, 5,762,938 and 5,766,599 (e.g., DNA encoding antigens from rabies, Hepatitis B, JEV, YF, Dengue, measles, pseudorabies, Epstein-Barr, HSV, HIV, SIV, EHV, BHV, HCMV, canine  
25 parvovirus, equine influenza, FeLV, FHV, Hantaan, C. tetani, avian influenza, mumps, NDV, inter alia); U.S. Pat. Nos 5,503,834 and 5,759,841 (e.g., Morbillivirus, e.g., measles F, hemagglutinin, inter alia); U.S. Pat. No 4,722,848 (e.g., HSV tk, HSV glycoproteins, e.g., gB, gD, influenza HA, Hepatitis B, e.g., HBsAg, inter alia); U.S. Pat. Nos 5,514,375, 5,744,140 and 5,744,141 (e.g., flavivirus structural proteins); U.S. Pat. No. 5,766,598 (e.g.,  
30 Lentivirus antigens such as immunodeficiency virus antigens, inter alia); U.S. Pat. Nos. 5,658,572 and 5,641,490 (e.g., IBDV antigens, inter alia); WO 94/16716 (e.g., cytokine

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and/or tumor associated antigens, inter alia); U.S. Pat. Nos. 5,688,920, and 5,529,780 (e.g., canine herpesvirus antigens), PCT publication WO 96/3941 (e.g., cytomegalovirus antigens); and U.S. Pat. Nos. 5,756,101 and 5,766,597 (Plasmodium antigens).

## 5 Variants of the target antigen

According to the invention, a cell-mediated immune response against a target antigen on a target cell of a subject is induced by the administration to the subject of a polynucleotide encoding a variant of the target antigen. A variant of the target antigen is preferably (i) a polypeptide which is homologous to, and/or a portion of, the target antigen;  
10 and (ii) which is not secreted from the cell, nor expressed on the extracellular side of cellular membrane of, the cell in which it was synthesized. The variant is preferably biologically inactive and/or prevented from being secreted or exposed on the extracellular side of the cell surface. A variant of the target antigen can also be fused to, or co-expressed with another polypeptide, e.g., a known immunogenic eukaryotic, prokaryotic or viral  
15 product.

The variant is preferably sufficiently related to the target antigen to ensure that the CD8+ cell-mediated response resulting from the administration of a polynucleotide encoding the variant into a subject, is directed to the target antigen. Thus, generally, the variant polypeptide shares a strong amino acid sequence homology with at least a portion of  
20 the target antigen. Preferably, the variant polypeptide has at least about 70% amino acid sequence homology of identity; more preferably at least about 80%; at least about 90%; at least about 95%; and even more preferably at least about 98 or 99% homology or identity.

The variant polypeptide is preferably not secreted, nor expressed on the extracellular side of the cellular membrane of the cell in which it was synthesized. Thus the variant may  
25 be a cytoplasmic or cytosolic protein. It can also be a properly modified nuclear, membrane, or secreted protein. The goal is to avoid the presence of any soluble form of the variant polypeptide, resulting from being either secreted or shed from the membrane, which would induce a humoral immune response against the target antigen. Thus, the production of antibodies against the target antigen is avoided by the use of a variant that will not  
30 appear in soluble form outside the transfected cell. Accordingly, the variant may be a form of the target polypeptide that is devoid of a signal peptide, i.e., a peptide which targets



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peptides being synthesized to the endoplasmic reticulum to be directed to the membrane or secreted. The location of signal peptides in known proteins is usually known in the art. There are also several algorithms that predict the location of sequences encoding signal peptides ("signal sequences"). Examples of such algorithms can be found at

- 5 <http://mbcf.dfci.harvard.edu/docs/Pedro.html>. Other domains of the target protein may also be deleted to keep the expressed variant in the cytosol. For example, in cases in which the target antigen is a receptor type protein, the transmembrane and/or cytoplasmic domain(s) of the antigen may be deleted. The location of such domains for known proteins is usually known. In addition, algorithms exist for the prediction of the localization of such domains.
- 10 These can also be found at <http://mbcf.dfci.harvard.edu/docs/Pedro.html>.

- In a preferred embodiment, the variant polypeptide is biologically inactive (but it is capable of inducing a cell-mediated immune response), to avoid the occurrence of any negative effects that might result from the expression of the variant polypeptide in cells of a subject or in *in vitro* cultured cells. Thus, it is desirable that the administration of a
- 15 polynucleotide encoding a variant polypeptide to a subject does not adversely affect the subject's health, such as by having a toxic effect on cells. Since the function of many target antigens, e.g., tumor-associated or tissue specific antigens, may be unknown, their synthesis and release by cells of the subject *in vivo* may lead to serious side effects. Furthermore, expression of a biologically functional protein may alter dendritic cell viability, change
- 20 their migration pattern or their ability to provide co-stimulation to T cells. A variant can be biologically inactive when it comprises only a portion of the target antigen, which does not include a biologically active domain of the target antigen, such as a portion of a cytoplasmic domain which associates with other proteins or DNA. Thus, if the target antigen is a receptor type molecule, using a variant that does not include the cytoplasmic
- 25 domain ensures that signals that would normally be transmitted through the cytoplasmic domain will be absent from the cell in which the variant of the target antigen is expressed.

- Furthermore, the smaller the variant polypeptide will be, the less likely it is that it will have a biological function. A variant of a target antigen can also be created by the introduction of one or more mutations in the target antigen or portion thereof. The mutation
- 30 can be an insertion, deletion or substitution of one or more amino acids. Mutation in polynucleotides encoding the target antigen can be made according to methods known in

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the art. Various methods exist for screening for biologically inactive polypeptides. For example, tests of biological activity and toxicity of variant polypeptides can be done *in vitro* as well as in mice or other laboratory animals. The particular test used will depend on the wild-type activity of the target peptide (i.e., testing for those variants which are depleted of that particular activity).

A variant of a target antigen can also be created by constructing polynucleotide sequences that encode for known immunogenic prokaryotic, eukaryotic or viral products that can be fused to, or co-expressed with the target antigen. Such immunogenic sequences are well known, and include, e.g., portions of keyhole limpet hemocyanin. Expression of such known immunogenic sequences will bypass existing tolerance to target antigen at the CD4+ T cell level by stimulating CD4+T cells reactive to said immunogenic sequences and by providing "by-stander" help.

Peptides that are presented by MHC class I complexes vary from 8-16 amino acids (Paul, "Fundamental Immunology", 2<sup>nd</sup> ed., 1989, Raven). Thus, preferred variants of target antigens are at least 8 amino acids long. They may also be at least 10 amino acids long, or at least 13, 14, 15 or 16 amino acids long. Other variants may also be at least about 20, 25, 30, 35, 40, 50 or 100 amino acids long. There is generally no upper limit of the size of the variant. As discussed above, the shorter the variant, the less likely it is to have a biological activity. However, the longer the variant is, the more likely it is to have one or more antigenic epitopes.

The variant may also be chosen such as not to include any B cell epitope. As described below, methods for identifying B cell epitopes in polypeptides are known in the art. Thus, preferred variants are those including one or more T cell epitopes, but no B cell epitope.

In an illustrative embodiment, the target antigen is a receptor-type molecule, e.g., PSMA, a variant of the target antigen may include only a portion of the extracellular domain, not including a signal peptide nor a transmembrane domain, and may optionally contain one or more mutations to ensure that the variant is biologically inactive.

The amino acid sequence of human PSMA is represented in Figure 1. The sequence is identical to SEQ ID NO: 2 of patent No. 5,538,866 by Israeli et al. (GenBank Accession No. AAB144401) and is set forth as SEQ ID NO: 2. The full length nucleotide sequence of

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the human PSMA cDNA encoding SEQ ID NO: 2 is set forth as SEQ ID NO: 1. This sequence corresponds to the nucleotide sequence set forth as SEQ ID NO: 1 in patent No. 5,538,866 by Israeli et al. (GenBank Accession No. I23794). The transmembrane domain corresponds to amino acids 20 to 43 of SEQ ID NO: 2. The cytoplasmic domain  
5 corresponds to amino acids 1 to 19 of SEQ ID NO: 2. The extracellular domain of PSMA corresponds to amino acids 44 to 750. Preferred variants of human PSMA that can be used according to the method of the invention include amino acids 44-750 of SEQ ID NO: 2 or portions thereof that are sufficient for permitting to be presented within the context of MHC class I and optionally MHC class II. Vectors and plasmids encoding the PSMA portion  
10 corresponding to amino acids 44-750 are further described in the Examples.

Another preferred PSMA variant for use in the invention corresponds to all, or a portion, of the splice variant of human PSMA corresponding to amino acids 58 to 750 of SEQ ID NO: 2, described in U.S. patent No. 5,935,818 by Israeli et al. This natural splice variant does not contain a transmembrane domain or a cytoplasmic domain, and could thus  
15 be used in the instant invention without further modification.

Yet other preferred human PSMA variants comprise amino acids 61-750 of SEQ ID NO: 2 (Form A); amino acids 55-750 of SEQ ID NO: 2 (Form B); amino acids 76-750 of SEQ ID NO: 2 (Form C); or amino acids 94-750 of SEQ ID NO: 2 (Form D). Thus, in preferred embodiments, a plasmid encoding the extracellular portion of human PSMA  
20 comprises nucleotides 391-2511 of SEQ ID NO: 1. Plasmids encoding a portion of the extracellular domain of human PSMA may comprise or consist of, or consist of about, nucleotides 433-2511 of SEQ ID NO: 1 (alternative splice variant); nucleotides 424-2511 of SEQ ID NO: 1 (Form B); nucleotides 442-2511 of SEQ ID NO: 1 (Form A); nucleotides 487-2511 of SEQ ID NO: 1 (Form C); or nucleotides 541-2511 of SEQ ID NO: 1 (Form D).  
25 It will be understood that shorter fragments, in particular fragments which do not extend to nucleotide 2511 are also within the scope of the invention.

Yet other preferred human PSMA variants comprise a portion of the extracellular domain, such as a portion containing at least 8, 10, 12, 15, 20, 25, 30, 40, 50, 100, 200 or 300 amino acids of SEQ ID NO: 2. Variants preferably consist of a portion of the  
30 extracellular domain of human PSMA, optionally fused to one or more heterologous sequences.

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Although less preferred, also within the scope of the invention are portions of the cytoplasmic domain with or without portions of the transmembrane and/or the extracellular domain.

Preferred variants of other human tumor antigens are as follows. A preferred  
5 variant of the human PSA antigen, is a form of the antigen in which the signal sequence is deleted. It is set forth in SEQ ID NO: 4, and is encoded by the nucleotide sequence set forth in SEQ ID NO: 3.

A preferred variant of the human CEA antigen is a form that is deleted of its signal sequence and is set forth in SEQ ID NO: 6 and encoded by the nucleotide sequence of SEQ  
10 ID NO: 5.

A preferred variant of human Her2 is set forth in SEQ ID NO: 8 and is encoded by the nucleotide sequence of SEQ ID NO: 7.

A truncated version of MAGE-12 that can be used according to the invention has the amino acid sequence set forth in SEQ ID NO: 10 and is encoded by the nucleotide  
15 sequence set forth in SEQ ID NO: 9. A truncated version of MAGE-6 that can be used according to the invention has the amino acid sequence set forth in SEQ ID NO: 12 and is encoded by the nucleotide sequence set forth in SEQ ID NO: 11.

A truncated version of MART-1 that can be used according to the invention has the amino acid sequence set forth in SEQ ID NO: 14 and is encoded by the nucleotide sequence  
20 set forth in SEQ ID NO: 13.

In each of SEQ ID Nos: 3, 5, 7, 9, 11, and 13, the Kozak sequence and start codon correspond to nucleotides 1-9.

Other preferred variants may be derived from polypeptides which are encoded by polymorphic alleles, or are mutated forms, of the target polypeptide which are naturally-  
25 occurring in cells other than the target cells. Variants may also be derived from genes, which are allogeneic or xenogeneic relative to the target antigen. It has been shown that expression of a xenogeneic form of a protein overcomes tolerance of the protein (J. Immun. (1997) 159:3113). Thus, variants may be prepared from allogeneic or xenogeneic genes, provided they are sufficiently similar to the target antigen that the ensuing cellular immune  
30 response is directed against the target antigen. Allogeneic or xenogeneic genes encoding

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the target antigen may be publicly available. Alternatively, the genes can be isolated by known methods, e.g., hybridization under low stringency conditions or PCR using degenerate primers.

In another embodiment, the invention provides a nucleic acid encoding a fusion protein of an antigen against which an immune response is desired and a peptide which enhances immune responses (immunogenic peptides). Such peptides are well known in the art. These can be viral or prokaryotic, and include keyhole limpet hemocyanin (KLH) and tetanus toxin. In another embodiment, the immunogenic peptide is administered as a separate protein.

One method that can be used to increase the likelihood of a portion of a target antigen to become associated with MHC class I genes in the APCs is to target the polypeptide to proteasomes. A proteasome is composed of up to 24 protein subunits that form a cylindrical complex, whose function is the degradation of cytosolic proteins that are tagged for turnover by covalent linkage to a small protein called ubiquitin. When a cytosolic protein becomes "ubiquitinated," it gains access to the proteasomal enzymatic activity, resulting in the degradation of the protein. It has been reported that at least certain protein antigens require ubiquitination before they can be presented to class I-restricted cells. Thus, tagging the variant polypeptide for ubiquitination increases the likelihood of the variant to be degraded, allowing fragments thereof to gain access to the exocytic pathway where the fragments can associate with MHC class I molecules and migrate to the cell surface for presentation to T cells. Polypeptides can be targeted for ubiquitination by fusing them with a domain that is recognized by ubiquitination enzymes, as described, e.g., in Winston et al. (1999) Curr. Biol. 9: 1180.

Another method for targeting proteins for degradation involves the use of heat shock proteins (Hsps), which, in particular, are known to be involved in carrying peptides from proteasomes to Class I molecules. In an illustrative embodiment, the method includes increasing the amount of Hsps in antigen presenting cells, such as professional antigen presenting cells and tumor cells, e.g., by cotransfecting antigen presenting cells with a nucleic acid encoding an Hsp. Alternatively, a nucleic acid encoding a fusion protein between an Hsp and an antigen against which an immune response is desired. Similarly,

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the cellular level of other chaperones may also be increased in a cell to enhance antigen presentation within the context of MHC class I (see, *infra*).

Also within the scope of the invention are polynucleotides encoding more than one variant. For example, where a cancer cell expresses more than one tumor antigen, variants  
5 deriving from two or more tumor antigens can be encoded by the polynucleotide for use according to the invention. Similarly variants from two or more microbial antigens may be encoded by the polynucleotide of the invention. These variants may be expressed as fusion proteins. The two or more variant polypeptides may be from the same pathogenic intracellular microorganism or from different ones, where the cells of the host are infected  
10 with more than one microorganism.

To be able to use the smallest variant possible, such as to reduce its likelihood of having a biological activity, or to specifically include several T cell epitopes, it may be desirable to know the location of potential T cell epitopes in a target antigen. Tumor-derived T cell epitopes of human tumor types (i.e., melanoma, ovarian, and breast  
15 carcinoma) are described, e.g., in Storkus, W. J., et al., *Biologic Therapy of Cancer* 2<sup>nd</sup> ed. DeVita, V. T., et al., editors. J. B. Lippincott Co., Philadelphia, Pa. 66-77 (1995). In addition, several methods exist for predicting the MHC class I epitopes of target antigens, such as methods in which peptides bound to MHC class I complexes are isolated and sequenced. Two principal methods have been used to isolate such peptides. In one of the  
20 two methods total cellular extraction of such peptides is carried out in pH 2.0 trifluoroacetic acid ("TFA"). This method results in cell cytolysis and release of total cytosolic peptides, only a fraction of which are actually class I-related. This method also typically employs protease inhibitors since cell cytolysis results in the release of proteolytic enzymes that can alter or destroy peptides of potential interest. See, Rotzschke, O., et al., *Nature* 348:252  
25 (1990), and Falk, K., et al., *Nature* 348:248 (1990). The second isolation method entails acid denaturation of immunoaffinity purified class I-peptide complexes. By contrast with the first method, the second method of peptide isolation is highly class I selective, and even class I allele specific since monoclonal antibodies directed against individual class I allotypes can be used to immunopurify class I complexes. By this latter approach, the  
30 majority of known class I-bound peptide sequence data has been acquired. See, for example, Van Bleek, G. M., et al., *Nature* 348:213 (1990); Rotzschke, O., et al., *Science*

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249:283 (1990); Madden, D. R., et al., *Nature* 353:326 (1991); Jardetzky, T. S., et al., *Nature* 351:290 (1991); and Nikolic-Zugic, J., et al., *Immunol Rev.* 10:54 (1991). Another method for identifying T cell epitopes is disclosed in U.S. 6,077,519, which describes methods for isolation and use of T cell epitopes eluted from viable cells in vaccines for treating cancer patients.

Another method for identifying T cell epitope can be derived from the following method, which has been used to identify B cell epitopes. This method includes the use of synthetic peptides have been used for "epitope mapping" to identify immunodominant determinants or epitopes on the surface of proteins for the development of new vaccines and diagnostics. Epitope mapping employs a series of overlapping peptides corresponding to regions on the protein of interest to identify sites which participate in antibody immunogenic determinant interaction. Commonly, epitope mapping employs peptides of relatively short length to precisely detect linear determinants. A fast method of epitope mapping known as PEPSCAN is based on the simultaneous synthesis of hundreds of overlapping peptides, of lengths of 8 to 14 amino acids, coupled to solid supports. The coupled peptides are tested for their ability to bind antibodies. The PEPSCAN approach is effective in localizing linear determinants, but not for the identification of epitopes needed for mimicry of discontinuous effector sites such as the HIV receptor/coreceptor binding site (Meloan et al., *Ann Biol. Clin.*, 1991; 49:231-242). An alternative method relies on a set of nested and overlapping peptides of multiple lengths ranging from 15 to 60 residues. These longer peptides can be reliably but laboriously synthesized by a series of independent solid-phase peptide syntheses, rather than by the rapid and simultaneous PEPSCAN syntheses. The resulting set of nested and overlapping peptides can then be used for analyses of antibody binding, to identify long peptides which best present immunodominant determinants, including simple discontinuous epitopes. This method is exemplified by the studies of Wang for the mapping of immunodominant sites from HTLV I/II (U.S. Pat. No. 5,476,765) and HCV (U.S. Pat. No. 5,106,726); and it was used for the selection of a precise position on the gp120 sequence for optimal presentation of an HIV neutralizing epitope (Wang et al., *Science*, 1991; 254:285-288).

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Cytotoxic T cell assays can be conducted by methods known in the art, which include, e.g., measurement of the amount of a labeled substance released from the cells targeted for lysis by the cytotoxic T cells.

Professional antigen presenting cells and precursors thereof for use, e.g., in cytotoxic T cell assays can be obtained according to methods well known in the art, and described, e.g., in U.S. patent No. 5,788,963. Dendritic cells can also be immortalized as described, e.g., in WO 94/28113.

Cell lines expressing specific target antigens, such as tumor associated antigens for testing T cytotoxic activity against target antigens, can be prepared by transfecting an expression vector encoding the target antigen in a cell line. Expression of the target antigen can be demonstrated by Northern blot analysis and/or Western blots using an antibody reactive against the target antigen. Numerous cell lines expressing target antigens are also available from the ATCC. For example, the ATCC deposit number of cell lines from various carcinomas, e.g., bladder, colon, breast, melanoma, and renal carcinoma, are listed in U.S. patent No. 6,093,399.

Polynucleotides of the invention can be isolated by hybridization under stringent or low stringency conditions. Thus, for example, to isolate variants of a target antigen, such as allogeneic or xenogeneic variants, a nucleic acid encoding a target antigen can be used as a probe to screen a cDNA library. Appropriate stringency conditions which promote DNA hybridization, for example, 6.0 x sodium chloride/sodium citrate (SSC) at about 45°C, followed by a wash of 2.0 x SSC at 50°C, are known to those skilled in the art or can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0 x SSC at 50°C to a high stringency of about 0.2 x SSC at 50°C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22°C, to high stringency conditions at about 65°C. Both temperature and salt may be varied, or temperature of salt concentration may be held constant while the other variable is changed.

To isolate a biologically inactive form of a target antigen for use as a variant in the invention, several methods can be used. For example, an analysis of the amino acid sequence of the antigen will reveal the location of specific biologically active domains and



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sequences, e.g., phosphorylation sites, SH2 or SH3 sites. The analysis may be performed by comparison of the amino acid sequence of the target antigen with that of other proteins, e.g., by using BLAST. Alternatively, algorithms known in the art can be used which predict the location of biologically active domains. Examples of such algorithms can be found at <http://mbcf.dfci.harvard.edu/docs/Pedro.html>.

In addition, libraries of fragments of the target antigen can be prepared and screened for those fragments, which lack biological activity. A variety of techniques are known in the art for generating such libraries, including chemical synthesis. In one embodiment, a library of coding sequence fragments can be generated by (i) treating a double stranded PCR fragment of target antigen coding sequence with a nuclease under conditions wherein nicking occurs only about once per molecule; (ii) denaturing the double stranded DNA; (iii) renaturing the DNA to form double stranded DNA which can include sense/antisense pairs from different nicked products; (iv) removing single stranded portions from reformed duplexes by treatment with S1 nuclease; and (v) ligating the resulting fragment library into an expression vector. By this exemplary method, an expression library can be derived which codes for N-terminal, C-terminal and internal fragments of various sizes.

A wide range of techniques are known in the art for screening gene products of combinatorial libraries made by point mutations or truncation, and for screening cDNA libraries for gene products having a certain property. Such techniques will be generally adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of homologs of a target antigen. The most widely used techniques for screening large libraries typically comprises cloning the library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial DNA fragments under conditions in which the absence of detection of a desired activity facilitates relatively easy isolation of the vector encoding the cDNA. Assays used for detecting biologically inactive fragments will usually be chosen based on the biological activity of the wild type target antigen, and will be amenable to high throughput analysis as necessary to screen large numbers of sequences of a target antigen created by combinatorial mutagenesis techniques.

A nucleic acid of the invention can also be linked to a stretch of CpG dinucleotides. It has been demonstrated that the immunogenicity of plasmid DNA is enhanced by short

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sequences that contain CpG dinucleotides in particular base contexts (see, e.g., Klinman et al. (1999) Vaccine 17:19 and Roman et al. (1997) Nat. Med. 3:849). It is believed that these sequences act as adjuvants. Instead of adding such short stretches (e.g., about 10, 16, 20, 30, 40, 50 or 100 nucleotides long) to a polynucleotide encoding the variant antigen, the  
5 sequence encoding the variant itself can be modified to increase the number of CpG dinucleotides.

Polynucleotides of the invention, expression vectors, and methods of delivery

The polynucleotides of the invention, i.e., encoding a variant of a target antigen,  
10 may be either a DNA or RNA sequence. The polynucleotide encoding a variant of a target antigen is preferably operably linked to all necessary transcriptional and translational regulatory elements, such as a promoter, enhancer and polyadenylation sequence. Regulatory sequences are art-recognized and are described, e.g., in Goeddel; Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA  
15 (1990). In a preferred embodiment, the promoter is a constitutive promoter, e.g., a strong viral promoter, e.g., CMV promoter. The promoter can also be cell- or tissue-specific, that permits substantial transcription of the DNA only in predetermined cells, e.g., in professional antigen presenting cells, such as the dendritic cell-specific CD11c promoter described in Brocker T, J. Leuk. Biology 66:331-335, 1999. The promoter can also be an  
20 inducible promoter, e.g., a metallothionein promoter. Other inducible promoters include those that are controlled by the inducible binding, or activation, of a transcription factor, e.g., as described in U.S. patent Nos. 5,869,337 and 5,830,462 by Crabtree et al., describing small molecule inducible gene expression (a genetic switch); International patent applications PCT/US94/01617, PCT/US95/10591, PCT/US96/09948 and the like, as well as  
25 in other heterologous transcription systems such as those involving tetracyclin-based regulation reported by Bujard et al., generally referred to as an allosteric "off-switch" described by Gossen and Bujard (Proc. Natl. Acad. Sci. U.S.A. (1992) 89:5547) and in U.S. Patents 5,464,758; 5,650,298; and 5,589,362 by Bujard et al. Other inducible transcription systems involve steroid or other hormone-based regulation.

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The polynucleotide of the invention together with all necessary transcriptional and translational control sequences is referred to herein as "construct of the invention" or "transgene of the invention."

The polynucleotide of the invention may also be introduced into the cell in which it is to be expressed together with another DNA sequence (which may be on the same or a different DNA molecule as the polynucleotide of the invention) coding for another agent. Exemplary agents are further described below. In one embodiment, the DNA encodes a polymerase for transcribing the DNA, and may comprise recognition sites for the polymerase and the injectable preparation may include an initial quantity of the polymerase.

In certain instances, it may be preferred that the polynucleotide is translated for a limited period of time so that the polypeptide delivery is transitory. This can be achieved, e.g., by the use of an inducible promoter.

The polynucleotides used in the present invention may also be produced in part or in total by chemical synthesis, e.g., by the phosphoramidite method described by Beaucage and Carruthers, Tetra. Letts., 22:1859-1862 (1981) or the triester method according to the method described by Matteucci et al., J. Am. Chem. Soc., 103:3185 (1981), and may be performed on commercial automated oligonucleotide synthesizers. A double-stranded fragment may be obtained from the single stranded product of chemical synthesis either by synthesizing the complementary strand and annealing the strand together under appropriate conditions or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

The polynucleotide of the invention operably linked to all necessary transcriptional and translational regulation elements can be injected as naked DNA into a subject or contacted *in vitro* with professional antigen presenting cells (further described below). In a preferred embodiment, the polynucleotide of the invention and necessary regulatory elements are present in a plasmid or vector. Thus, the polynucleotide of the invention may be DNA, which is itself non-replicating, but is inserted into a plasmid, which may further comprise a replicator. The DNA may be a sequence engineered so as not to integrate into the host cell genome.

Preferred vectors for use according to the invention are expression vectors, i.e., vectors that allow expression of a nucleic acid in a cell vectors. Preferred expression

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vectors are those which contain both prokaryotic sequences, to facilitate the propagation of the vector in bacteria, and one or more eukaryotic transcription units that are expressed in eukaryotic cells. The pcDNAI/amp, pcDNAI/neo, pRc/CMV, pSV2gpt, pSV2neo, pSV2-dhfr, pTk2, pRSVneo, pMSG, pSVT7, pko-neo and pHyg derived vectors are examples of mammalian expression vectors suitable for transfection of eukaryotic cells. Some of these vectors are modified with sequences from bacterial plasmids, such as pBR322, to facilitate replication and drug resistance selection in both prokaryotic and eukaryotic cells.

Alternatively, derivatives of viruses such as the bovine papillomavirus (BPV-1), or Epstein-Barr virus (pHEBo, pREP-derived and p205) can be used for transient expression of proteins in eukaryotic cells. The various methods employed in the preparation of the plasmids and transformation of host organisms are well known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells, as well as general recombinant procedures, see Molecular Cloning A Laboratory Manual, 2<sup>nd</sup> Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989) Chapters 16 and 17.

Any means for the introduction of polynucleotides into mammals, human or non-human, may be adapted to the practice of this invention for the delivery of the various constructs of the invention into the intended recipient. In one embodiment of the invention, the DNA constructs are delivered to cells by transfection, i.e., by delivery of "naked" DNA or in a complex with a colloidal dispersion system. A colloidal system includes macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. The preferred colloidal system of this invention is a lipid-complexed or liposome-formulated DNA. In the former approach, prior to formulation of DNA, e.g., with lipid, a plasmid containing a transgene bearing the desired DNA constructs may first be experimentally optimized for expression (e.g., inclusion of an intron in the 5' untranslated region and elimination of unnecessary sequences (Felgner, et al., Ann NY Acad Sci 126-139, 1995). Formulation of DNA, e.g. with various lipid or liposome materials, may then be effected using known methods and materials and delivered to the recipient mammal. See, e.g., Canonico et al, Am J Respir Cell Mol Biol 10:24-29, 1994; Tsan et al, Am J Physiol 268; Alton et al., Nat Genet. 5:135-142, 1993 and U.S. patent No. 5,679,647 by Carson et al. Colloidal dispersion systems.

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The targeting of liposomes can be classified based on anatomical and mechanistic factors. Anatomical classification is based on the level of selectivity, for example, organ-specific, cell-specific, and organelle-specific. Mechanistic targeting can be distinguished based upon whether it is passive or active. Passive targeting utilizes the natural tendency of liposomes to distribute to cells of the reticulo-endothelial system (RES) in organs which contain sinusoidal capillaries. Active targeting, on the other hand, involves alteration of the liposome by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein, or by changing the composition or size of the liposome in order to achieve targeting to organs and cell types other than the naturally occurring sites of localization.

The surface of the targeted delivery system may be modified in a variety of ways. In the case of a liposomal targeted delivery system, lipid groups can be incorporated into the lipid bilayer of the liposome in order to maintain the targeting ligand in stable association with the liposomal bilayer. Various linking groups can be used for joining the lipid chains to the targeting ligand. Naked DNA or DNA associated with a delivery vehicle, e.g., liposomes, can be administered to several sites in a subject (see below).

In a preferred method of the invention, the DNA constructs are delivered using viral vectors. The transgene may be incorporated into any of a variety of viral vectors useful in gene therapy, such as recombinant retroviruses, adenovirus, adeno-associated virus (AAV), and herpes simplex virus-1, or recombinant bacterial or eukaryotic plasmids. While various viral vectors may be used in the practice of this invention, AAV- and adenovirus-based approaches are of particular interest. Such vectors are generally understood to be the recombinant gene delivery system of choice for the transfer of exogenous genes *in vivo*, particularly into humans. The following additional guidance on the choice and use of viral vectors may be helpful to the practitioner. As described in greater detail below, such embodiments of the subject expression constructs are specifically contemplated for use in various *in vivo* and *ex vivo* gene therapy protocols.

#### A. Adenoviral vectors

A viral gene delivery system useful in the present invention utilizes adenovirus-derived vectors. Knowledge of the genetic organization of adenovirus, a 36 kB, linear and

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double-stranded DNA virus, allows substitution of a large piece of adenoviral DNA with foreign sequences up to 8 kB. In contrast to retrovirus, the infection of adenoviral DNA into host cells does not result in chromosomal integration because adenoviral DNA can replicate in an episomal manner without potential genotoxicity. Also, adenoviruses are structurally stable, and no genome rearrangement has been detected after extensive amplification. Adenovirus can infect virtually all epithelial cells regardless of their cell cycle stage. So far, adenoviral infection appears to be linked only to mild disease such as acute respiratory disease in the human.

Adenovirus is particularly suitable for use as a gene transfer vector because of its mid-sized genome, ease of manipulation, high titer, wide target-cell range, and high infectivity. Both ends of the viral genome contain 100-200 base pair (bp) inverted terminal repeats (ITR), which are cis elements necessary for viral DNA replication and packaging. The early (E) and late (L) regions of the genome contain different transcription units that are divided by the onset of viral DNA replication. The E1 region (E1A and E1B) encodes proteins responsible for the regulation of transcription of the viral genome and a few cellular genes. The expression of the E2 region (E2A and E2B) results in the synthesis of the proteins for viral DNA replication. These proteins are involved in DNA replication, late gene expression, and host cell shut off (Renan (1990) Radiotherap. Oncol. 19:197). The products of the late genes, including the majority of the viral capsid proteins, are expressed only after significant processing of a single primary transcript issued by the major late promoter (MLP). The MLP (located at 16.8 m.u.) is particularly efficient during the late phase of infection, and all the mRNAs issued from this promoter possess a 5' tripartite leader (TL) sequence which makes them preferred mRNAs for translation.

The genome of an adenovirus can be manipulated such that it encodes a gene product of interest, but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle (see, for example, Berkner et al., (1988) BioTechniques 6:616; Rosenfeld et al., (1991) Science 252:431-434; and Rosenfeld et al., (1992) Cell 68:143-155). Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are well known to those skilled in the art. Recombinant adenoviruses can be advantageous in certain circumstances in that they are not capable of infecting nondividing cells and can be used to infect a wide variety of cell

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types, including airway epithelium (Rosenfeld et al., (1992) cited supra), endothelial cells (Lemarchand et al., (1992) PNAS USA 89:6482-6486), hepatocytes (Herz and Gerard, (1993) PNAS USA 90:2812-2816) and muscle cells (Quantin et al., (1992) PNAS USA 89:2581-2584).

5           Adenovirus vectors have also been used in vaccine development (Grunhaus and Horwitz (1992) Siminar in Virology 3:237; Graham and Prevec (1992) Biotechnology 20:363). Experiments in administering recombinant adenovirus to different tissues include trachea instillation (Rosenfeld et al. (1991) ; Rosenfeld et al. (1992) Cell 68:143), muscle injection (Ragot et al. (1993) Nature 361:647), peripheral intravenous injection  
10 (Herz and Gerard (1993) Proc. Natl. Acad. Sci. U.S.A. 90:2812), and stereotactic inoculation into the brain (Le Gal La Salle et al. (1993) Science 254:988).

          Furthermore, the virus particle is relatively stable and amenable to purification and concentration, and as above, can be modified so as to affect the spectrum of infectivity. Additionally, adenovirus is easy to grow and manipulate and exhibits broad host range *in vitro* and *in vivo*. This group of viruses can be obtained in high titers, e.g.,  $10^9 - 10^{11}$   
15 plaque-forming unit (PFU)/ml, and they are highly infective. The life cycle of adenovirus does not require integration into the host cell genome. The foreign genes delivered by adenovirus vectors are episomal, and therefore, have low genotoxicity to host cells. No side effects have been reported in studies of vaccination with wild-type adenovirus (Couch  
20 et al., 1963; Top et al., 1971), demonstrating their safety and therapeutic potential as *in vivo* gene transfer vectors. Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases) relative to other gene delivery vectors (Berkner et al., supra; Haj-Ahmand and Graham (1986) J. Virol. 57:267). Most replication-defective adenoviral vectors currently in use and therefore favored by the present invention are  
25 deleted for all or parts of the viral E1 and E3 genes but retain as much as 80% of the adenoviral genetic material (see, e.g., Jones et al., (1979) Cell 16:683; Berkner et al., supra; and Graham et al., in Methods in Molecular Biology, E.J. Murray, Ed. (Humana, Clifton, NJ, 1991) vol. 7. pp. 109-127). Expression of the inserted polynucleotide of the invention can be under control of, for example, the E1A promoter, the major late promoter (MLP) and  
30 associated leader sequences, the viral E3 promoter, or exogenously added promoter sequences.

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Other than the requirement that the adenovirus vector be replication defective, or at least conditionally defective, the nature of the adenovirus vector is not believed to be crucial to the successful practice of the invention. The adenovirus may be of any of the 42 different known serotypes or subgroups A-F. Adenovirus type 5 of subgroup C is the preferred starting material in order to obtain the conditional replication-defective adenovirus vector for use in the method of the present invention. This is because Adenovirus type 5 is a human adenovirus about which a great deal of biochemical and genetic information is known, and it has historically been used for most constructions employing adenovirus as a vector. As stated above, the typical vector according to the present invention is replication defective and will not have an adenovirus E1 region. Thus, it will be most convenient to introduce the nucleic acid of interest at the position from which the E1 coding sequences have been removed. However, the position of insertion of the polynucleotide or construct on the invention (also referred to as "nucleic acid of interest") in a region within the adenovirus sequences is not critical to the present invention. For example, it may also be inserted in lieu of the deleted E3 region in E3 replacement vectors as described previously by Karlsson et. al. (1986) or in the E4 region where a helper cell line or helper virus complements the E4 defect.

A preferred helper cell line is 293 (ATCC Accession No. CRL1573). This helper cell line, also termed a "packaging cell line" was developed by Frank Graham (Graham et al. (1987) J. Gen. Virol. 36:59-72 and Graham (1977) J. General Virology 68:937-940) and provides E1A and E1B in trans. However, helper cell lines may also be derived from human cells such as human embryonic kidney cells, muscle cells, hematopoietic cells or other human embryonic mesenchymal or epithelial cells. Alternatively, the helper cells may be derived from the cells of other mammalian species that are permissive for human adenovirus. Such cells include, e.g., Vero cells or other monkey embryonic mesenchymal or epithelial cells.

Adenoviruses can also be cell type specific, i.e., infect only restricted types of cells and/or express a transgene only in restricted types of cells. For example, the viruses comprise a gene under the transcriptional control of a transcription initiation region specifically regulated by target host cells, as described e.g., in U.S. Patent No. 5,698,443, by Henderson and Schuur, issued December 16, 1997. Thus, replication competent



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adenoviruses can be restricted to certain cells by, e.g., inserting a cell specific response element to regulate a synthesis of a protein necessary for replication, e.g., E1A or E1B.

DNA sequences of a number of adenovirus types are available from Genbank. For example, human adenovirus type 5 has GenBank Accession No.M73260. The adenovirus DNA sequences may be obtained from any of the 42 human adenovirus types currently identified. Various adenovirus strains are available from the American Type Culture Collection, Rockville, Maryland, or by request from a number of commercial and academic sources. A transgene as described herein may be incorporated into any adenoviral vector and delivery protocol, by restriction digest, linker ligation or filling in of ends, and ligation.

Adenovirus producer cell lines can include one or more of the adenoviral genes E1, E2a, and E4 DNA sequence, for packaging adenovirus vectors in which one or more of these genes have been mutated or deleted are described, e.g., in PCT/US95/15947 (WO 96/18418) by Kadan et al.; PCT/US95/07341 (WO 95/346671) by Kovesdi et al.; PCT/FR94/00624 (WO94/28152) by Imler et al.; PCT/FR94/00851 (WO 95/02697) by Perrocaudet et al., PCT/US95/14793 (WO96/14061) by Wang et al.

#### B. AAV Vectors

Yet another viral vector system useful for delivery of the subject polynucleotides is the adeno-associated virus (AAV). Adeno-associated virus is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle. (For a review, see Muzyczka et al., Curr. Topics in Micro. and Immunol. (1992) 158:97-129).

AAV has not been associated with the cause of any disease. AAV is not a transforming or oncogenic virus. AAV integration into chromosomes of human cell lines does not cause any significant alteration in the growth properties or morphological characteristics of the cells. These properties of AAV also recommend it as a potentially useful human gene therapy vector.

AAV is also one of the few viruses that may integrate its DNA into non-dividing cells, e.g., pulmonary epithelial cells, and exhibits a high frequency of stable integration (see for example Flotte et al., (1992) Am. J. Respir. Cell. Mol. Biol. 7:349-356; Samulski et

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al., (1989) J. Virol. 63:3822-3828; and McLaughlin et al., (1989) J. Virol. 62:1963-1973). Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate. Space for exogenous DNA is limited to about 4.5 kb. An AAV vector such as that described in Tratschin et al., (1985) Mol. Cell. Biol. 5:3251-3260 can be used to introduce  
5 DNA into cells. A variety of nucleic acids have been introduced into different cell types using AAV vectors (see for example Hermonat et al., (1984) PNAS USA 81:6466-6470; Tratschin et al., (1985) Mol. Cell. Biol. 4:2072-2081; Wondisford et al., (1988) Mol. Endocrinol. 2:32-39; Tratschin et al., (1984) J. Virol. 51:611-619; and Flotte et al., (1993) J. Biol. Chem. 268:3781-3790).

10 The AAV-based expression vector to be used typically includes the 145 nucleotide AAV inverted terminal repeats (ITRs) flanking a restriction site that can be used for subcloning of the transgene, either directly using the restriction site available, or by excision of the transgene with restriction enzymes followed by blunting of the ends, ligation of appropriate DNA linkers, restriction digestion, and ligation into the site between the  
15 ITRs. The capacity of AAV vectors is about 4.4 kb. The following proteins have been expressed using various AAV-based vectors, and a variety of promoter/enhancers: neomycin phosphotransferase, chloramphenicol acetyl transferase, Fanconi's anemia gene, cystic fibrosis transmembrane conductance regulator, and granulocyte macrophage colony-stimulating factor (Kotin, R.M., Human Gene Therapy 5:793-801, 1994, Table I). A  
20 transgene incorporating the various DNA constructs of this invention can similarly be included in an AAV-based vector. As an alternative to inclusion of a constitutive promoter such as CMV to drive expression of the polynucleotide of interest, an AAV promoter can be used (ITR itself or AAV p5 (Flotte, et al. J. Biol.Chem. 268:3781-3790, 1993)).

Such a vector can be packaged into AAV virions by reported methods. For  
25 example, a human cell line such as 293 can be co-transfected with the AAV-based expression vector and another plasmid containing open reading frames encoding AAV rep and cap (which are obligatory for replication and packaging of the recombinant viral construct) under the control of endogenous AAV promoters or a heterologous promoter. In the absence of helper virus, the rep proteins Rep68 and Rep78 prevent accumulation of the  
30 replicative form, but upon superinfection with adenovirus or herpes virus, these proteins permit replication from the ITRs (present only in the construct containing the transgene)

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and expression of the viral capsid proteins. This system results in packaging of the transgene DNA into AAV virions (Carter, B.J., *Current Opinion in Biotechnology* 3:533-539, 1992; Kotin, R.M, *Human Gene Therapy* 5:793-801, 1994)). Typically, three days after transfection, recombinant AAV is harvested from the cells along with adenovirus and  
5 the contaminating adenovirus is then inactivated by heat treatment.

Methods to improve the titer of AAV can also be used to express the polynucleotide of the invention in an AAV virion. Such strategies include, but are not limited to: stable expression of the ITR-flanked transgene in a cell line followed by transfection with a second plasmid to direct viral packaging; use of a cell line that expresses AAV proteins  
10 inducibly, such as temperature-sensitive inducible expression or pharmacologically inducible expression. Alternatively, a cell can be transformed with a first AAV vector including a 5' ITR, a 3' ITR flanking a heterologous gene, and a second AAV vector which includes an inducible origin of replication, e.g., SV40 origin of replication, which is capable of being induced by an agent, such as the SV40 T antigen and which includes DNA  
15 sequences encoding the AAV rep and cap proteins. Upon induction by an agent, the second AAV vector may replicate to a high copy number, and thereby increased numbers of infectious AAV particles may be generated (see, e.g, U.S. Patent No. 5,693,531 by Chiorini et al., issued December 2, 1997. In yet another method for producing large amounts of recombinant AAV, a chimeric plasmid is used which incorporate the Epstein Barr Nuclear  
20 Antigen (EBNA) gene, the latent origin of replication of Epstein Barr virus (oriP) and an AAV genome. These plasmids are maintained as a multicopy extra-chromosomal elements in cells, such as in 293 cells. Upon addition of wild-type helper functions, these cells will produce high amounts of recombinant AAV (U.S. Patent 5,691,176 by Lebkowski et al., issued Nov. 25, 1997). In another system, an AAV packaging plasmid is provided that  
25 allows expression of the rep gene, wherein the p5 promoter, which normally controls rep expression, is replaced with a heterologous promoter (U.S. Patent 5,658,776, by Flotte et al., issued Aug. 19, 1997). Additionally, one may increase the efficiency of AAV transduction by treating the cells with an agent that facilitates the conversion of the single stranded form to the double stranded form, as described in Wilson et al., WO96/39530.

30 AAV stocks can be produced as described in Hermonat and Muzyczka (1984) PNAS 81:6466, modified by using the pAAV/Ad described by Samulski et al. (1989) J.

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Virol. 63:3822. Concentration and purification of the virus can be achieved by reported methods such as banding in cesium chloride gradients, as was used for the initial report of AAV vector expression *in vivo* (Flotte, et al. J.Biol. Chem. 268:3781-3790, 1993) or chromatographic purification, as described in O'Riordan et al., WO97/08298.

5           Methods for *in vitro* packaging AAV vectors are also available and have the advantage that there is no size limitation of the DNA packaged into the particles (see, U.S. Patent No. 5,688,676, by Zhou et al., issued Nov. 18, 1997). This procedure involves the preparation of cell free packaging extracts.

10           For additional detailed guidance on AAV technology which may be useful in the practice of the subject invention, including methods and materials for the incorporation of a transgene, the propagation and purification of the recombinant AAV vector containing the transgene, and its use in transfecting cells and mammals, see e.g. Carter et al, US Patent No. 4,797,368 (10 Jan 1989); Muzyczka et al, US Patent No. 5,139,941 (18 Aug 1992); Lebkowski et al, US Patent No. 5,173,414 (22 Dec 1992); Srivastava, US Patent No. 15 5,252,479 (12 Oct 1993); Lebkowski et al, US Patent No. 5,354,678 (11 Oct 1994); Shenk et al, US Patent No. 5,436,146(25 July 1995); Chatterjee et al, US Patent No. 5,454,935 (12 Dec 1995), Carter et al WO 93/24641 (published 9 Dec 1993), and Natsoulis, U.S. Patent No. 5,622,856 (April 22, 1997). Further information regarding AAVs and the adenovirus or herpes helper functions required can be found in the following articles. Berns and 20 Bohensky (1987), "Adeno-Associated Viruses: An Update", Advanced in Virus Research, Academic Press, 33:243-306. The genome of AAV is described in Laughlin et al. (1983) "Cloning of infectious adeno-associated virus genomes in bacterial plasmids", Gene, 23: 65-73. Expression of AAV is described in Beaton et al. (1989) "Expression from the Adeno-associated virus p5 and p19 promoters is negatively regulated in trans by the rep 25 protein", J. Virol., 63:4450-4454. Construction of rAAV is described in a number of publications: Tratschin et al. (1984) "Adeno-associated virus vector for high frequency integration, expression and rescue of genes in mammalian cells", Mol. Cell. Biol., 4:2072-2081; Hermonat and Muzyczka (1984) "Use of adeno-associated virus as a mammalian DNA cloning vector: Transduction of neomycin resistance into mammalian tissue culture 30 cells", Proc. Natl. Acad. Sci. USA, 81:6466-6470; McLaughlin et al. (1988) "Adeno-associated virus general transduction vectors: Analysis of Proviral Structures", J. Virol.,

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62:1963-1973; and Samulski et al. (1989) "Helper-free stocks of recombinant adeno-associated viruses: normal integration does not require viral gene expression", J. Virol., 63:3822-3828. Cell lines that can be transformed by rAAV are those described in Lebkowski et al. (1988) "Adeno-associated virus: a vector system for efficient introduction and integration of DNA into a variety of mammalian cell types", Mol. Cell. Biol., 8:3988-3996. "Producer" or "packaging" cell lines used in manufacturing recombinant retroviruses are described in Dougherty et al. (1989) J. Virol., 63:3209-3212; and Markowitz et al. (1988) J. Virol., 62:1120-1124.

#### 10 C. Hybrid Adenovirus-AAV Vectors

Hybrid Adenovirus-AAV vectors represented by an adenovirus capsid containing a nucleic acid comprising a portion of an adenovirus, and 5' and 3' ITR sequences from an AAV which flank a selected transgene under the control of a promoter. See e.g. Wilson et al, International Patent Application Publication No. WO 96/13598. This hybrid vector is characterized by high titer transgene delivery to a host cell and the ability to stably integrate the transgene into the host cell chromosome in the presence of the rep gene. This virus is capable of infecting virtually all cell types (conferred by its adenovirus sequences) and stable long term transgene integration into the host cell genome (conferred by its AAV sequences).

The adenovirus nucleic acid sequences employed in the this vector can range from a minimum sequence amount, which requires the use of a helper virus to produce the hybrid virus particle, to only selected deletions of adenovirus genes, which deleted gene products can be supplied in the hybrid viral process by a packaging cell. For example, a hybrid virus can comprise the 5' and 3' inverted terminal repeat (ITR) sequences of an adenovirus (which function as origins of replication). The left terminal sequence (5') sequence of the Ad5 genome that can be used spans bp 1 to about 360 of the conventional adenovirus genome (also referred to as map units 0-1) and includes the 5' ITR and the packaging/enhancer domain. The 3' adenovirus sequences of the hybrid virus include the right terminal 3' ITR sequence which is about 580 nucleotides (about bp 35,353- end of the adenovirus, referred to as about map units 98.4-100).

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The AAV sequences useful in the hybrid vector are viral sequences from which the rep and cap polypeptide encoding sequences are deleted and are usually the cis acting 5' and 3' ITR sequences. Thus, the AAV ITR sequences are flanked by the selected adenovirus sequences and the AAV ITR sequences themselves flank a selected transgene. The preparation of the hybrid vector is further described in detail in published PCT application entitled "Hybrid Adenovirus-AAV Virus and Method of Use Thereof", WO 96/13598 by Wilson et al.

For additional detailed guidance on adenovirus and hybrid adenovirus-AAV technology which may be useful in the practice of the subject invention, including methods and materials for the incorporation of a transgene, the propagation and purification of recombinant virus containing the transgene, and its use in transfecting cells and mammals, see also Wilson et al, WO 94/28938, WO 96/13597 and WO 96/26285, and references cited therein.

#### 15 D. Retroviruses

The retroviruses are a group of single-stranded RNA viruses characterized by an ability to convert their RNA to double-stranded DNA in infected cells by a process of reverse-transcription (Coffin (1990) Retroviridae and their Replication" In Fields, Knipe ed. Virology. New York: Raven Press). The resulting DNA then stably integrates into cellular chromosomes as a provirus and directs synthesis of viral proteins. The integration results in the retention of the viral gene sequences in the recipient cell and its descendants. The retroviral genome contains three genes, gag, pol, and env that code for capsial proteins, polymerase enzyme, and envelope components, respectively. A sequence found upstream from the gag gene, termed psi , functions as a signal for packaging of the genome into virions. Two long terminal repeat (LTR) sequences are present at the 5' and 3' ends of the viral genome. These contain strong promoter and enhancer sequences and are also required for integration in the host cell genome (Coffin (1990), supra).

In order to construct a retroviral vector, a nucleic acid of interest is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication-defective. In order to produce virions, a packaging cell line containing the gag, pol, and env genes but without the LTR and psi components is constructed (Mann et al. (1983) Cell

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33:153). When a recombinant plasmid containing a human cDNA, together with the retroviral LTR and psi sequences is introduced into this cell line (by calcium phosphate precipitation for example), the psi sequence allows the RNA transcript of the recombinant plasmid to be packaged into viral particles, which are then secreted into the culture media  
5 (Nicolas and Rubenstein (1988) "Retroviral Vectors", In: Rodriguez and Denhardt ed. Vectors: A Survey of Molecular Cloning Vectors and their Uses. Stoneham:Butterworth; Temin, (1986) "Retrovirus Vectors for Gene Transfer: Efficient Integration into and Exprssion of Exogenous DNA in Vertebrate Cell Genome", In: Kucherlapati ed. Gene Transfer. New York: Plenum Press; Mann et al., 1983, supra). The media containing the  
10 recombinant retroviruses is then collected, optionally concentrated, and used for gene transfer. Retroviral vectors are able to infect a broad variety of cell types. However, integration and stable expression require the division of host cells (Paskind et al. (1975) Virology 67:242).

A major prerequisite for the use of retroviruses is to ensure the safety of their use,  
15 particularly with regard to the possibility of the spread of wild-type virus in the cell population. The development of specialized cell lines (termed "packaging cells") which produce only replication-defective retroviruses has increased the utility of retroviruses for gene therapy, and defective retroviruses are well characterized for use in gene transfer for gene therapy purposes (for a review see Miller, A.D. (1990) Blood 76:271). Thus,  
20 recombinant retrovirus can be constructed in which part of the retroviral coding sequence (gag, pol, env) has been replaced by nucleic acid encoding a protein of the present invention, e.g., a transcriptional activator, rendering the retrovirus replication defective. The replication defective retrovirus is then packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques. Protocols for  
25 producing recombinant retroviruses and for infecting cells *in vitro* or *in vivo* with such viruses can be found in Current Protocols in Molecular Biology, Ausubel, F.M. et al., (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art. A preferred retroviral vector is a pSR MSVtkNeo  
30 (Muller et al. (1991) Mol. Cell Biol. 11:1785 and pSR MSV(XbaI) (Sawyers et al. (1995) J. Exp. Med. 181:307) and derivatives thereof. For example, the unique BamHI sites in both of these vectors can be removed by digesting the vectors with BamHI, filling in with

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Klenow and religating to produce pSMTN2 and pSMTX2, respectively, as described in PCT/US96/09948 by Clackson et al. Examples of suitable packaging virus lines for preparing both ecotropic and amphotropic retroviral systems include Crip, Cre, 2 and Am.

5           Retroviruses have been used to introduce a variety of genes into many different cell types, including neural cells, epithelial cells, endothelial cells, lymphocytes, myoblasts, hepatocytes, bone marrow cells, *in vitro* and/or *in vivo* (see for example Eglitis et al., (1985) Science 230:1395-1398; Danos and Mulligan, (1988) PNAS USA 85:6460-6464; Wilson et al., (1988) PNAS USA 85:3014-3018; Armentano et al., (1990) PNAS USA  
10 87:6141-6145; Huber et al., (1991) PNAS USA 88:8039-8043; Ferry et al., (1991) PNAS USA 88:8377-8381; Chowdhury et al., (1991) Science 254:1802-1805; van Beusechem et al., (1992) PNAS USA 89:7640-7644; Kay et al., (1992) Human Gene Therapy 3:641-647; Dai et al., (1992) PNAS USA 89:10892-10895; Hwu et al., (1993) J. Immunol. 150:4104-4115; U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286; PCT Application WO  
15 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573).

          Furthermore, it has been shown that it is possible to limit the infection spectrum of retroviruses and consequently of retroviral-based vectors, by modifying the viral packaging proteins on the surface of the viral particle (see, for example PCT publications  
20 WO93/25234, WO94/06920, and WO94/11524). For instance, strategies for the modification of the infection spectrum of retroviral vectors include: coupling antibodies specific for cell surface antigens to the viral env protein (Roux et al., (1989) PNAS USA 86:9079-9083; Julan et al., (1992) J. Gen Virol 73:3251-3255; and Goud et al., (1983) Virology 163:251-254); or coupling cell surface ligands to the viral env proteins (Neda et  
25 al., (1991) J. Biol. Chem. 266:14143-14146). Coupling can be in the form of the chemical cross-linking with a protein or other variety (e.g. lactose to convert the env protein to an asialoglycoprotein), as well as by generating fusion proteins (e.g. single-chain antibody/env fusion proteins). This technique, while useful to limit or otherwise direct the infection to certain tissue types, and can also be used to convert an ecotropic vector in to an  
30 amphotropic vector.



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## E. Other Viral Systems

Other viral vector systems that can be used to deliver a polynucleotide of the invention have been derived from herpes virus, e.g., Herpes Simplex Virus (U.S. Patent No. 5,631,236 by Woo et al., issued May 20, 1997 and WO 00/08191 by Neurovex), vaccinia virus (Ridgeway (1988) Ridgeway, "Mammalian expression vectors," In: Rodriguez R L, Denhardt D T, ed. Vectors: A survey of molecular cloning vectors and their uses. Stoneham: Butterworth,; Baichwal and Sugden (1986) "Vectors for gene transfer derived from animal DNA viruses: Transient and stable expression of transferred genes," In: Kucherlapati R, ed. Gene transfer. New York: Plenum Press; Coupar et al. (1988) Gene, 68:1-10), and several RNA viruses. Preferred viruses include an alphavirus, a poxivirus, an arena virus, a vaccinia virus, a polio virus, and the like. They offer several attractive features for various mammalian cells (Friedmann (1989) Science, 244:1275-1281 ; Ridgeway, 1988, supra; Baichwal and Sugden, 1986, supra; Coupar et al., 1988; Horwich et al.(1990) J.Virol., 64:642-650).

With the recent recognition of defective hepatitis B viruses, new insight was gained into the structure-function relationship of different viral sequences. *In vitro* studies showed that the virus could retain the ability for helper-dependent packaging and reverse transcription despite the deletion of up to 80% of its genome (Horwich et al., 1990, supra). This suggested that large portions of the genome could be replaced with foreign genetic material. The hepatotropism and persistence (integration) were particularly attractive properties for liver-directed gene transfer. Chang et al. recently introduced the chloramphenicol acetyltransferase (CAT) gene into duck hepatitis B virus genome in the place of the polymerase, surface, and pre-surface coding sequences. It was cotransfected with wild-type virus into an avian hepatoma cell line. Culture media containing high titers of the recombinant virus were used to infect primary duckling hepatocytes. Stable CAT gene expression was detected for at least 24 days after transfection (Chang et al. (1991) Hepatology, 14:124A).

Administration of the polynucleotides of the invention

In one embodiment of the invention, the polynucleotide is administered to the subject in need thereof. The polynucleotide can be introduced into muscle tissue, tissues of

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skin, brain, lung, liver, spleen or blood. The preparation can be injected into the vertebrate by a variety of routes, which may be intradermally, subdermally, intramuscularly, intrathecally, or intravenously, or it may be placed within cavities of the body. In a preferred embodiment, the polynucleotide is injected intramuscularly. In still other  
5   embodiments, the preparation comprising the polynucleotide is impressed into the skin. Transdermal administration is also contemplated, as is inhalation.

In a preferred embodiment, the polynucleotide is injected into a tissue of the subject that is rich in professional antigen presenting cells, e.g., dendritic cells. Thus, the polynucleotide is preferably administered to the skin or mucosa, where approximately 1%  
10   to 2% of the cell population is comprised of antigen presenting cells. A preferred route of administration is intradermally.

Due to the existence of cross-priming, i.e., the transfer of MHC-peptide- or HSP-peptide- complexes from one cell to another, it is not necessary that the nucleic acid be transfected only into professional antigen presenting cells. It is believed that the transfer of  
15   MHC-or HSP-peptides complexes occurs by small vesicles (icosomes) derived from cell membranes that are shed from cells, which then fuse with other cells, e.g., professional antigen presenting cells. Accordingly, a nucleic acid that is taken up and expressed in a cell other than a professional antigen presenting cell, and which is, e.g., a myocyte, may be expressed and presented within MHC class I on the cell surface. By cross-priming, this  
20   peptide can be taken up by MHC class I or class II complexes on professional antigen presenting cells and thereby activate T cells.

A regimen of immunization of a subject can include administration of a single type of vector (viral vector or plasmid vector) or of different types of vectors. The regimen may comprise administration of a single dose of vector. However, it is preferable that the  
25   administration comprises several administrations, e.g., every other day, every third day, every fifth day, once a week, one a month, one every two or three months or once every 6 months or every year. In a preferred embodiment, a nucleic acid of the invention is administered to a subject once per three weeks for nine weeks, and then once every three months. The regimen may be interrupted depending on the state of the clinical state and the  
30   response of the subject, or it may be carried on for several years.

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In a preferred embodiment, the regimen includes first administering to a patient one or more doses of a plasmid DNA, e.g., XC PSMA, and then administering to the patient one or more doses of a viral vector DNA, e.g., Ad5-XC PSMA. Without wanting to be limited to a specific mechanism of action, it is believed that initial administration(s) of a plasmid vector rather than of a viral vector, and subsequent administration of viral vector is beneficial at least in part because most of the recipient subject's immune responses will be directed toward the few proteins expressed by the plasmid, thus predominantly against the antigen expressed therefrom. On the contrary, if a viral vector is administered before a plasmid vector, the recipient subject's immune responses will be directed against various other antigens, and will, essentially be "diluted." In fact, it is known that viral DNA and proteins encoded thereby have adjuvant properties. Accordingly, in a preferred embodiment, a subject to be treated receives two doses of a plasmid vector and one dose of a viral vector at 3-week intervals, followed by reimmunizations at three months intervals alternating between a plasmid or a viral vector. Other regimens are set forth in the Examples.

The interval between the first administrations may be one week. After the initial administrations, the viral and/or the plasmid vector can be administered every few months, e.g., every three months, such as to boost the immune response. In an embodiment in which DNA encoding a costimulatory molecule is co-administered, the later DNA is preferably coadministered together with the DNA encoding the variant. The coding sequence can be on the same or on different DNA molecules. In embodiments in which a growth factor or hormone is co-administered, this factor can be administered simultaneously or consecutively to a patient. Various regimens and particular doses of the reagents are further described in the examples.

The nucleic acids of the invention can be administered to a subject in conjunction with another type of treatment. In a preferred embodiment, the method of the invention is used in conjunction with surgery. In an even more preferred embodiment, a subject may receive a nucleic acid of the invention after surgery that removed at least a portion, and preferably most of, the cancer cells.

Generally the DNA or viral particles of the invention are transferred to a biologically compatible solution or pharmaceutically acceptable delivery vehicle, such as

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sterile saline, or other aqueous or non-aqueous isotonic sterile injection solutions or suspensions, numerous examples of which are well known in the art, including Ringer's, phosphate buffered saline, or other similar vehicles. Delivery of the transgene as naked DNA; as lipid-, liposome-, or otherwise formulated DNA; or as a recombinant viral vector  
5 can then be carried out *in vivo* or *in vitro* (e.g., *ex vivo*). This can be accomplished by various means, including nebulization/inhalation or by instillation via bronchoscopy. Recently, recombinant adenovirus encoding CFTR was administered via aerosol to human subjects in a phase I clinical trial. Vector DNA and CFTR expression were clearly detected in the nose and airway of these patients with no acute toxic effects (Bellonet al., Human  
10 Gene Therapy, 8(1):15-25, 1997).

Preferably, the DNA or recombinant virus is administered in sufficient amounts to transfect cells within the desired tissue of the recipient, and provide sufficient levels of transgene expression to provide for expression of the polynucleotide of the invention in the desired cells, preferably at a level providing therapeutic benefit without undue adverse  
15 effects.

Optimal dosages of DNA or virus depends on a variety of factors, as discussed previously, and may thus vary somewhat from patient to patient. Again, therapeutically effective doses of viruses are considered to be in the range of about 20 to about 50 ml of saline solution containing concentrations of from about  $1 \times 10^7$  to about  $1 \times 10^{10}$  pfu of  
20 virus/ml, e.g. from  $1 \times 10^8$  to  $1 \times 10^9$  pfu of virus/ml.

Toxicity and therapeutic efficacy of ligands and constructs or viral particles (generally referred to as "reagents") can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD<sub>50</sub> (the dose lethal to 50% of the population) and the ED<sub>50</sub> (the dose therapeutically effective in 50% of  
25 the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD<sub>50</sub>/ED<sub>50</sub>. Reagents which exhibit large therapeutic indices are preferred. While reagents that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such reagents to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby,  
30 reduce side effects.

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The data obtained from cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such reagents lies preferably within a range of circulating concentrations that include the  $ED_{50}$  with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any reagent used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the  $IC_{50}$  (i.e., the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

Pharmaceutical compositions for use in accordance with the present invention may be formulated in conventional manner using one or more physiologically acceptable carriers or excipients. Thus, the compounds and their physiologically acceptable salts and solvates may be formulated for administration by, for example, injection, inhalation or insufflation (either through the mouth or the nose) or oral, buccal, parenteral or rectal administration.

For such therapy, the reagents of the invention can be formulated for a variety of loads of administration, including systemic and topical or localized administration.

Techniques and formulations generally may be found in Remington's Pharmaceutical Sciences, Meade Publishing Co., Easton, PA. For systemic administration, injection is preferred, including intramuscular, intravenous, intraperitoneal, and subcutaneous. For injection, the reagents of the invention can be formulated in liquid solutions, preferably in physiologically compatible buffers such as Hank's solution or Ringer's solution. In addition, the compounds may be formulated in solid form and redissolved or suspended immediately prior to use. Lyophilized forms are also included.

For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g., pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g., magnesium

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stearate, talc or silica); disintegrants (e.g., potato starch or sodium starch glycolate); or wetting agents (e.g., sodium lauryl sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g., sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g., lecithin or acacia); non-aqueous vehicles (e.g., almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (e.g., methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate.

Preparations for oral administration may be suitably formulated to give controlled release of the active ingredient. For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner. For administration by inhalation, the reagents for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of e.g. gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The reagents may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

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The reagents may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the reagents may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the reagents may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration bile salts and fusidic acid derivatives. In addition, detergents may be used to facilitate permeation. Transmucosal administration may be through nasal sprays or using suppositories. For topical administration, the oligomers of the invention are formulated into ointments, salves, gels, or creams as generally known in the art.

In clinical settings, the gene delivery systems for the polynucleotid or construction of the invention can be introduced into a patient by any of a number of methods, each of which is familiar in the art. For instance, a pharmaceutical preparation of the gene delivery system can be introduced systemically, e.g. by intravenous injection, and specific transduction of the protein in the target cells occurs predominantly from specificity of transfection provided by the gene delivery vehicle, cell-type or tissue-type expression due to the transcriptional regulatory sequences controlling expression of the receptor gene, or a combination thereof. In other embodiments, initial delivery of the recombinant gene is more limited with introduction into the animal being quite localized. For example, the gene delivery vehicle can be introduced by catheter (see U.S. Patent 5,328,470) or by stereotactic injection (e.g. Chen et al. (1994) PNAS 91: 3054-3057).

The pharmaceutical preparation of the gene therapy construct can consist essentially of the gene delivery system in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene

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delivery system can be produced intact from recombinant cells, e.g. retroviral vectors, the pharmaceutical preparation can comprise one or more cells which produce the gene delivery system.

5 The compositions may, if desired, be presented in a pack or dispenser device, which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

10 In another embodiment of the invention, the polynucleotide of the invention is first administered to a professional antigen presenting cell (professional APC) or precursor thereof, which is then administered to the subject. The professional APC can be from the same subject or from another subject, or different source. In a preferred embodiment, professional APCs, such as dendritic cells, are obtained from a subject, contacted with the polynucleotide of the invention and administered back to the subject. Dendritic cells and precursors thereof can be obtained from peripheral blood mononuclear cells (PBMCs) as  
15 described in the Examples, or they can be obtained and stored as described, e.g., in WO93/20185 by Steinman et al. or in U.S. patent No. 5,788,963.

In yet another embodiment, professional APCs, e.g., obtained from a subject, are contacted with a polynucleotide of the invention, and then contacted *ex vivo* with a population of cells comprising CD8<sup>+</sup> T cells under conditions allowing activation of CD8<sup>+</sup>  
20 T cells. The state of activation of the CD8<sup>+</sup> T cells can be monitored by, e.g., performing cytotoxicity tests. The cells are then administered to the subject in need thereof. The whole population of cells can be administered, or purified portions thereof, e.g., the CD8<sup>+</sup> T cells.

Alternatively, the activated CD8<sup>+</sup>T cells can be contacted *ex vivo* with a population of cells comprising target cells, such as an organ or bone marrow to be transplanted into a  
25 subject. The cell population is then administered back to the subject after destruction of the target cells.

Also within the scope of the invention are methods for delivering variants of target peptides directly to cells, i.e., not through delivery of polynucleotides encoding the variants. Although entry of peptides into the exocytic pathway that delivers the peptide-MHC complex to the cell surface is usually required for presentation of peptides in  
30 association with MHC class I complexes, non-cellular-delivery vehicles for proteins, such



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as pH-sensitive liposomes, can over-come the requirement for endogenous synthesis *in vivo* (Nair, et al., J Exp Med, (1992), 175, 609-12, Nair, et al., J Virol, (1993), 67, 4062-9). Thus, the invention also provides methods in which variant polypeptides are administered to a subject in a form that facilitates their uptake by cells, e.g., liposomes.

5           In yet another embodiment, small variant peptides (8-11-mer) are administered to a subject. These synthetic peptides associate with class I molecules on the cell surface without the requirement for endogenous processing. When presented on the surface of an appropriate APC (such as a dendritic cell) they can then induce a primary CTL response (Stauss, et al., Proc Natl Acad Sci U S A, (1992), 89, 7871-5, Carbone, et al., J Exp Med, 10           (1988), 167, 1767-79). Clinical trials using peptides derived from tumor-associated antigens which can be loaded onto the MHC molecules of dendritic cells simply by incubation *in vitro* have recently been undertaken (see, e.g., Murphy et al. (1996) Prostate 29:371 and Tjoa et al. (1999) Prostate 40:125). However, frequently these CTL do not protect against challenge with pathogens that endogenously synthesize the protein from 15           which the peptide was derived because of their low T-cell receptor avidity (Speiser, et al., J Immunol, (1992), 149, 972-80) and because they induce reactivity with a single epitope of the target antigen.

#### Co-administration of other agents

20           To improve the induction of a cellular immune response against the polypeptide encoded by a polynucleotide of the invention administered to a subject, the polynucleotide encoding the polypeptide can be administered together with another agent. When the agent is a polynucleotide, it can be present on the same or on a separate DNA molecule from that encoding the polypeptide of the invention.

25           In one embodiment, the method of the invention further comprises co-administration of one or more cytokines, in particular an immune-enhancing cytokine, such as GM-CSF, IL-2, IL-4, IL-12, IL-15, IL-18 or polynucleotide encoding such. In order to initiate an immune response, an antigen must be presented to naive T cells by professional APCs. The most efficient APCs *in vivo* are the dendritic cells. These cells are present in tissues and, 30           when alerted by "danger signals," they mature, accumulate antigen and migrate to the regional lymph nodes. Such "danger signals" can be coadministered with a polynucleotide

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of the invention. A preferred "danger signal" is granulocyte-macrophage colony stimulating factor (GM-CSF), which has been shown to promote differentiation, proliferation, survival and migration of dendritic cells (Jones, et al., Eur J Clin Microbiol Infect Dis, (1994), 13, S47-53). The benefit of administering GM-CSF is also shown in  
5 the examples. Administration of recombinant human GM-CSF is well tolerated as described herein and in Armitage (1998) Blood 92:4491. Preferred doses of GM-CSF per administration include from about 10,000 I.U. to about 100,000 I.U., more preferably from about 10,000 to about 40,000 I.U.

In another embodiment, the polynucleotide is administered together with a  
10 polynucleotide encoding a costimulatory molecule, e.g., B7 (B7-1, B7-2, and/or B7-3). Effective stimulation of resting naïve T and memory T cells by an antigen presenting cell requires at least two separate signal, the first one of which is the antigen and the second that of a costimulatory molecule. The absence of the second signal results in T cell paralysis or death. Human B7-1 is also referred to as CD80 and human B7-2 as CD86. Both of these  
15 molecules have been cloned. As described in the examples, co-administration of a gene encoding a B7 molecule is beneficial in at least certain circumstances. Methods for administering polynucleotides encoding one or more B7 molecule and an antigen are also described, e.g., in U.S. patent 5,738,852 by Robinson et al.

In another embodiment, polynucleotides encoding MHC class I gene(s) are co-  
20 administered to the subject, such as to increase the number of MHC class I receptors on the surface of the target cell to increase the number of target antigens presented on the target cell. Progression of disease has been associated with loss or decrease of class I expression by cancer cells. A complete loss of HLA class I has been reported to occur in 34% of primary prostate cancers and up to 80% of lymph node metastases (Blades et al. Urology  
25 1995,46681-686). When individual allelic expression was assessed, the minimum estimate of down regulation was up to 85% in the primary prostate cancers and almost 100% in the metastases (Blades et al., supra). Thus, increasing the number of MHC class I and/or class II molecules on antigen presenting cells, in particular undesirable cells, may be advantageous in the method of the invention. In one embodiment, the sequence encoding  
30 the derivative of the target antigen and the sequence encoding the MHC class I gene(s) are not on the same nucleic acid, since these sequences are to be expressed in different cells:

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the sequence encoding the derivative of the target antigen is to be introduced into professional APCs, whereas the sequence encoding MHC class I genes is to be introduced into the undesirable cell. Genes encoding beta2-microglobulin can also be co-administered, either together with MHC class I genes, or on their own.

5           The density of MHC class I complexes on the surface of target cells can also be increased by contacting the target cells, e.g., by administering to the subject, agents which stimulate MHC class I complex formation or MHC class I and/or beta2-microglobulin expression in target cells. Examples of such agents include IFN-gamma.

          Additional proteins, or nucleic acids encoding such, that can be co-administered  
10   with a nucleic acid of the invention include those which are involved in the formation of peptide-MHC class I complexes and its trafficking to the cell surface, e.g., molecular chaperones. Co-administration of such molecules would enhance presentation of antigen by antigen presenting cells, e.g., dendritic cells. Exemplary proteins include proteins that are associated with or are part of TAP-Class I complexes. These proteins include Transport  
15   Associated Proteins (TAPs) , e.g., TAP-1 and TAP2, calnexin calreticulin, tapasin, and the Erp57 protein (see, e.g., Sadasvan et al. (1996) Immunity 5:103; Morrice and Powis (1998) Curr. Biol. 8: 713; Suh et al. (1996) J. Exp. Med. 184:337; Lindquist et al. (1998) EMBO J. 17:2186; and Hughes and Cresswell (1998) Curr. Biol. 8: 709). Calnexin and calreticulin are both lectins that are expressed predominantly in the ER. Erp57 is a chaperone with  
20   thiolreductase activity. Instead of introducing a nucleic acid encoding one or more of these proteins into cells, the level of expression of the endogenous proteins can also be increased. For example, the expression level of tapansin can be increased by interferon-gamma.

          In yet another embodiment, the nucleic acid of the invention is co-administered with one or more heat shock proteins (Hsps) or nucleic acids encoding such. Hsps have been  
25   shown to participate in anti-tumor T cell responses, at least in part by increasing the number of peptide/MHC Class I complexes on the surface of tumor cells. This was shown, e.g., by stably transfecting clones of B16 melanoma cells, which are not effectively recognized by MHC class I-restricted CTL due to very low levels of MHC class I antigens on the surface, with DNA encoding human heat shock protein 72 (Hsp72), such that they constitutively  
30   express Hsp 72. It was found that these stable transfectants exhibited significantly increased levels of MHC class I antigens on their surface. This Hsp72-mediated up-

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regulation of surface MHC class I antigen represents an increase in the amount of functional MHC-peptide complexes as measured by conformation-dependent antibodies and recognition by MHC class I-restricted CTL. Moreover, it was also shown that mice immunized with Hsp72-expressing B16 cells, but not with control-transfected B16 cells, display significantly increased resistance to a subsequent challenge with live, wild-type B16. Thus, these data demonstrate that the immune recognition of tumor cells can be substantially enhanced by the suitable expression of a molecular chaperone. Accordingly, in certain embodiments of the invention it may be desirable to co-administer a nucleic acid encoding a Hsp, e.g., Hsp72.

It has also be shown that an Hsp70 acts as a chaperone in cross-priming, i.e., in transferring an MHC class I associated peptide from one cell to another. Thus, co-administration of Hsp70 or other chaperone capable of acting in this manner, or a nucleic acid encoding Hsp70 or other chaperone, would enhance cross-priming, thereby enhancing delivery of the antigen of the invention to the right target cells, i.e., professional antigen presenting cells. Cross-priming is in fact believed to be a principal mechanism by which plasmid DNA delivered to cells such as myocytes effectively shuttle Ag to professional antigen presenting cells to achieve CTL induction in vivo.

The polynucleotide of the invention may also be co-administered with a polynucleotide encoding a homing receptor, such that the APCs at the point of entry will serve as vehicles to deliver the polynucleotide to lymphatic organs and to mucosal tissues other than those at the point of entry. Other agents that can be co-administered include adhesion molecules, members of the tumor necrosis factors superfamily, e.g., TNF-alpha, TNF-beta, and Fas-ligand. Yet other agents for co-administration include CD40 ligand, CD54, CD50, and CD58. The amino acid sequence of these proteins is known in the art, as well as nucleotide sequences encoding these.

Another agent that can be co-administered to a subject or to cells *in vitro* include polynucleotides encoding a target antigen. As set forth above, cells, which do not express a target cell can be modified to become target cells by introducing in these cells a polynucleotide encoding a target antigen. Of course, even a cell, which naturally expresses a target antigen can further be modified to increase the number of target antigens that it expresses, or to express one or more other target antigens. Thus, a target cell can be

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contacted with a polynucleotide encoding a target antigen, such that the polynucleotide enters the cell and is expressed in the cell (e.g., transfected). Such a polynucleotide could be co-administered to a subject together with the polynucleotide encoding the variant of the target antigen.

5           It will be understood that, to increase the number of target antigens produced in a target cell, the target cells can also be contacted with an agent, which induces the expression of the target antigen, or otherwise increases the number of target antigens produced.

10           The present invention is further illustrated by the following examples which should not be construed as limiting in any way. The contents of all cited references including literature references, issued patents, published or non published patent applications as cited throughout this application are hereby expressly incorporated by reference. The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant  
15   DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); *DNA Cloning*, Volumes I and II (D. N. Glover ed., 1985); *Oligonucleotide Synthesis* (M. J. Gait ed., 1984); Mullis et al. U.S. Patent No: 4,683,195; *Nucleic Acid Hybridization* (B. D.  
20   Hames & S. J. Higgins eds. 1984); *Transcription And Translation* (B. D. Hames & S. J. Higgins eds. 1984); *Culture Of Animal Cells* (R. I. Freshney, Alan R. Liss, Inc., 1987); *Immobilized Cells And Enzymes* (IRL Press, 1986); B. Perbal, *A Practical Guide To Molecular Cloning* (1984); the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y.); *Gene Transfer Vectors For Mammalian Cells* (J. H. Miller and M. P. Calos eds.,  
25   1987, Cold Spring Harbor Laboratory); *Methods In Enzymology*, Vols. 154 and 155 (Wu et al. eds.), *Immunochemical Methods In Cell And Molecular Biology* (Mayer and Walker, eds., Academic Press, London, 1987); *Handbook Of Experimental Immunology*, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

### Examples

#### **Example 1: Preparation of a plasmid mammalian expression vector encoding the extracellular domain of PSMA**

5 cDNA of PSMA extracellular fragment (2118 bp) was obtained using total mRNA from the prostate tumor cell line LNCaP.FGC - CRL 1740 (ATCC). A PSMA-specific 3'-primer was used for reverse transcription of mRNA, which was performed using RT from avian myeloblastosis virus (Boehringer). The resulting cDNA was then amplified using High Fidelity PCR System (Boehringer) and the gel purified PCR product of expected  
10 length was cloned into pCR2.1 vector (Invitrogen). Two clones were selected and checked by DNA sequencing. The resulting construct contains the extracellular portion of PSMA, i.e., amino acids 44-750 of SEQ ID NO: 1 (without any mutation) with the NotI- Kozak sequence at its 5' end and SfuI site at its 3' end, which were introduced during the PCR amplification.

15 The above-described DNA fragment was then introduced into the mammalian expression vector pcDNA3.1 (Invitrogen) using the NotI-SfuI cloning sites. The vector provides human cytomegalovirus (CMV) immediate-early promoter/enhancer region permitting efficient, high-level expression of recombinant protein as well as 3' flanking region containing bovine growth hormone polyadenylation signal for efficient transcription  
20 termination and increasing the half life of the mRNA *in vivo*. The neomycin resistance gene was (NRG) was removed by digestion with NaeI endonuclease and ligation of the NRG-free fragment of the plasmid following gel purification.

The resulting pcDNA3.1 expression vector containing a sequence encoding the extracellular domain of human PSMA (referred to as "XC-PSMA") was deposited as  
25 Designation Number 203168 on August 28, 1998 at the American Type Culture Collection, 10801 University Blvd., Manassas, Virginia 20110.

PSMA-CD86 plasmid was constructed as follows. Th full length human CD86 cDNA was amplified from human monocytes of a helathy donor and cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA). The cDNA was then sub-cloned into a modified  
30 pcDNA 3.1 (Invitrogen, carlsbad, CA), in which the neomycin resistance gene was deleted. A portion of this plasmid containing the sequence encoding human CD86, human cytomegalovirus (CMB) immediate early promoter/enhancer located immediately upstream

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of the CD86 sequence, and bovine growth hormone (BGH) polyadenylation signal located immediately downstream of the CD86 sequence, was inserted into XC-PSMA plasmid between the BGH polyadenylation signal of PSMA and ht eColE1 origin of replication. Thus, both genes are controlled by the same regulatory sequences. This plasmid is referred to herein as "XC PSMA- CD86" plasmid.

**Example 2: Preparation of a replication-defective recombinant adenovirus Ad5-PSMA**

Ad5-PSMA recombinant adenovirus, comprising a sequence encoding the extracellular domain of PSMA under the control of the CMV promoter, was prepared using the kit available from Quantum Biotechnology Inc. The portion of the plasmid that contains the CMV promoter-PSMA fragment-PolyA signal was cut from pcDNA3.1 containing the extracellular domain of PSMA described above using BglII and SmaI restriction endonucleases. The resulting product was purified on an agarose gel and subcloned in the BglII-EcoRV cloning sites of the pAdBN transfer vector (Quantum Biotechnologies Inc., Montreal, Canada).

The transfer vector was then linearized with ClaI, and co-transfected with linearized Adenovirus DNA into 293A cells. The recombinant replication-defective adenovirus(which lacks E1 and E3) was purified three times and clones that were positive for PSMA expression were selected by immunoblotting. The positive clone was amplified in 293 cells and then purified on two successive CsCl gradients. Finally the purified virus was dialyzed against PBS-5% sucrose.

The replication-defective recombinant adenovirus Ad5-PSMA was deposited as Designation Number VR2362 on August 28, 1998 at the American Type Culture Collection, 10801 University Blvd., Manassas, Virginia 20110.

**Example 3: *In vitro* activation of CD8+ T cells by dendritic cells infected with Ad5-PSMA**

Peripheral blood mononuclear cells (PBMC) from healthy anonymous donors were isolated from freshly drawn blood by density centrifugation on Ficoll-Paque at 468 g at 22°C for 30 minutes. PBMC were resuspended in RPMI with 5% autologous serum

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(complete medium) culture medium at  $1 \times 10^6$  cells/ml and allowed to adhere onto  $175 \text{ cm}^2$  polystyrene tissue culture flask. The flasks are incubated at  $37^\circ\text{C}$  and shaken every 20 minutes during incubation. After 1 hrs at  $37^\circ\text{C}$ , non-adherent cells are removed and adherent cells are cultured in 30 ml medium containing 2 ng/ml granulocyte macrophage colony-stimulating factor GM-CSF (obtained from Immunex, Seattle, WA) and 4 ng/ml interleukin -4 (IL-4) (obtained from Sigma). Cells were cultured for 5 days, following which dendritic cells (DCs) were harvested by centrifugation and used for experiments following verification by light microscopy examination and flow-cytometry.

DCs were infected with the Ad5-PSMA recombinant adenovirus at a multiplicity of infection (MOI) of 100. Infection experiments were carried out in polypropylene tubes to prevent the adherence of the cells. 50  $\mu\text{l}$  of viral suspension was inoculated into 50  $\mu\text{l}$  of cell suspension ( $1.5 \times 10^6$  cells) in complete RPMI1640 medium containing 2% of autologous serum. After inoculation, the cells were incubated 90 min at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ , in complete RPMI-1640 medium containing 2% of autologous serum, then washed three times, and incubated in RPMI-1640 medium containing 10% of autologous serum for an additional 24h at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ . Expression of PSMA was tested by immunoblotting.

The results indicate that the efficiency of infection of DCs by the Ad5-PSMA recombinant adenovirus was 20%, i.e., 20% of the DCs has been infected by the recombinant adenovirus.

In another example, DCs were obtained from HLA-A2+ patients, infected with the Ad5-PSMA recombinant adenovirus, and cultured with autologous T cells in complete medium (CM) for 3 days at  $37^\circ\text{C}$ . T cells were harvested at the end of the incubation, CD8+ T cells were purified therefrom by negative depletion with antiCD4 antibodies and complement, and their cytotoxicity was tested.

The results indicate that the CD8+ T cells that had been stimulated by autologous DCs infected with Ad5-PSMA were cytotoxic against the prostate tumor cell line LNCaP.FGC (also of the HLA A2+ phenotype), but not against Jurkat (T leukemia) or U937 (myelomonocytic cell line) cells. In comparison, freshly separated T cells showed no cytotoxicity against any of the three cell lines.



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**Example 4: Improvement of prostate cancer in subjects having received a plasmid and/or recombinant adenovirus expressing the extracellular domain of human PSMA****Patient Treatment with Plasmid or Adenovirus****5 Study design:**

One group of seven patients received three injections of extracellular (XC) PSMA-DNA plasmid or XC PSMA- CD86 plasmid at the same dose (100 ug) at one-week intervals. Five patients (see Table I) also received 10,000 IU GM-CSF (Leukine; Immunex, Seattle, WA) at the site of the plasmid application immediately, 24, or 48 hours after the  
10 immunization. Additionally, two months later, these seven patients and a group of 2 new patients received three injections of the replication-deficient Ad5-PSMA recombinant adenovirus (also referred to as "Ad5-XC-PSMA") vaccine ( $5 \times 10^8$  PFUs per application) at on -week intervals. The plasmid was injected intradermally between the first and second toes of the right leg, or intramuscularly. The viral vaccine was administered intradermally  
15 in the navel area.

Constant monitoring of the clinical state and the vital signs was carried out for 2 hours after vaccination. If stable, the subject was allowed to leave the hospital. A brief follow-up visit occurred 24 (and 48 in the case of GM-CSF inoculation) hours later.

**Inclusion criteria:**

20 All patients signed an informed consent form before admission into the study. Data from monitoring visits were shared with the patients as the study proceeded, and the patients were reminded that they were free to withdraw from participation at any time. Only patients with advanced, hormone-resistant cancer or patients unable to find or administer hormone therapy were included into the study.

25 Patients with a history of another malignancy or with a serious active infection or with another illness were excluded from the study.

**Monitoring Studies:**

Standard laboratory tests included CBC, urinalysis, liver enzymes, antinuclear antibodies, erythrocyte sedimentation rate, PSA. Each patient had a pelvic CAT scan, chest  
30 radiograph and a cardiograph on entry and on week 20 (week 10 for the 2 patients

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immunized with virus only). Safety was defined as lack of untoward clinical or laboratory events, with particular attention to local and systemic reactions as well evidence of anti-nuclear antibody.

5        Additionally, analysis of CD/HLA DR<sup>+</sup>; CD4<sup>+</sup>; CD8<sup>+</sup>; CD3<sup>+</sup>/CD16<sup>+</sup>CD56<sup>+</sup>; CD3<sup>+</sup>; CD1<sup>+</sup>; CD25<sup>+</sup>; CD19<sup>+</sup> cells as well as CD4/CD8 ratio prior and following immunotherapy was performed by flow cytometry.

### *Results*

#### *Characteristics of participants:*

10        Nine men, ages between 49 and 69 with advanced adenocarcinoma of the prostate, were included in the study. Three patients had had a radical prostatectomy, 2 were in preparation for surgery, three were inoperable and one was operable but had other contraindications for surgery treatment. Two of the patients died due to advanced cancer disease.

#### *Safety monitoring results:*

15        The immunizations were well tolerated. No changes in vital signs occurred following injections or on follow-up visits.

20        Patients who received intradermal immunizations with plasmid had minor DTH-like reactions 24 hours following the third immunization. Patients NN 8 and 9 developed a DTH reaction 24 hours following each administration of the recombinant adenovirus. Patients NN I through 7 had no DTH-like reactions 24 hrs after the first immunization with the viral vector, but developed DTH after the second and third immunization. All DTH-like local reactions were mild and resolved within 72 hrs post immunization.

      Patient N 4 had a vesicular rash after the last viral immunization which was located on the back and which resolved in the next two days with no treatment.

25        Patient N 7 had a papular urticaria-like rash with small petechiae at the center which developed 24 hrs after the last plasmid immunization and which disappeared after the discontinuation of the antibiotic therapy he was receiving.

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No significant changes occurred in erythrocyte sedimentation rate, CBC, serum creatinine or other blood chemistries, or urinalysis. Serum liver chemistry values remained within normal range in all subjects.

5      No significant changes in the analysis of CD/HLA DR<sup>+</sup>; CD4<sup>+</sup>; CD8<sup>+</sup>; CD3<sup>-</sup> /CD16<sup>+</sup>CD56<sup>+</sup>; CD3<sup>+</sup>; CD1<sup>+</sup>; CD25<sup>+</sup>; CD19<sup>+</sup> cells as well as CD4/CD8 ratio prior and following immunotherapy were detected.

No subject developed abnormal vital signs following injection, no significant increase in antinuclear antibodies titers were observed, and anti-DNA antibody was not detected.

10      For PSA values, CAT-scans, bone scintigraphy or lymph node metastases before and after immunization see tables 1 and 2.

Patients were immunized initially three times at weekly intervals with PSMA plasmid. Two months later all patients but patient # 7 received three additional immunizations at weekly intervals with the recombinant adenovirus.

Table 1: Regimen and characteristics of patients 1-7 treated with Ad5-PSMA

Patient #	Stage of disease	Type of Immunization	Additional treatment	PSA(ng/ml) before after	CT scan before after	LN before after	Bone metastases before after	Side effects
1	T <sub>4</sub> N <sub>4</sub> M <sub>2</sub> inoperable	3x plsmd i.d. 3 x Ad5PSMA	orchiectomy Casodex	6.3 -	+++ +++	- +	++ ++	exitus
2	T <sub>2</sub> N <sub>2</sub> X <sub>0</sub> operable <sup>#</sup>	3x plsmd i.m. +GM-CSF 3 x Ad5PSMA	orchiectomy Androcur	14.38 0.28	+++ *	- -	- -	none
3	T <sub>4</sub> N <sub>4</sub> M <sub>2</sub> inoperable	3x plsmd i.d. 3 x Ad5PSMA	orchiectomy	33.0 0.04	+++ *	- -	- +	none
4	T <sub>4</sub> M <sub>2</sub> M <sub>2</sub> post BPH and TUR inoperable	3x plsmd i.d. +GM-CSF 3 x Ad5PSMA	orchiectomy (recently Fluconazole)	1.11 3.8	++ ++	- -	+ ++	none
5	T <sub>2-3</sub> N <sub>2</sub> M <sub>0</sub> in preparation for surgery	3x plsmd i.d. +GM-CSF 3 x Ad5PSMA	MAB	3.01 0.05	++ *	- -	- -	none
6	T <sub>3-4</sub> N <sub>2</sub> M <sub>2</sub> post TUR	3x plsmd i.d. +GM-CSF 3 x Ad5PSMA	orchiectomy MAB	1.6 0.04	+++ *	- -	- -	none
7	T <sub>4</sub> N <sub>4</sub> M <sub>2</sub> post radical prostatectomy metastases	3x plsmd i.m. +GM-CSF	MAB	100	+++ ++	** -	++	exitus skin rash ***

Legend:

CT scan: CAT scan to obtain an estimate of the size of the prostate gland.

PSA stands for prostate specific antigen and is an indication of the prognosis of the patient: a better prognosis is indicated by a lower PSA level.

Plsmid: stands for plasmid. Patients 1, 4, 5, and 7 received plasmid XC-PSMA, whereas patients 2, 3, and 6 received plasmid XC-PSMA-CD86.

LN refers to lymph node metastasis.

++,+++ : increase in the size of the prostate gland or presence of metastatic tumor post radical prostatectomy (patient # 7)

-;+: lack (-) or presence of bone metastases or lymph node engagement

\*: significant decrease in the size of the prostate gland

\*\* : Patient # 7. Lack of urine excretion from both urethers due to metastases prior to the immune therapy. Appearance of diuresis from the right kidney one month after the last immunization. Died due to mechanical ileus following blockade of the rectum and sigmoidum by metastases.

\*\*\*: Patient # 7 had a mild skin rash 24 hrs post the third plasmid application which disappeared after discontinuation of the concurrent antibiotic therapy.

#: Patient # 2 could not have surgery due to cardiovascular complications.

MAB: maximum androgen blockade with Zoladex, Casodex or Flucinome

orchiectomy: always bilateral

**Table 2: Patients who were immunized with recombinant adenovirus (Ad-PSMA) 3 times at weekly intervals:**

Patient #	Stage of disease	Type of immunization	Additional treatment	PSA(ng/ml) before	CT scan after	LN before	Bone after	Side after metastases	effects
8	T <sub>4</sub> N <sub>2</sub> M post radical prostatectomy metastases	3xAd5PSMA	MAB	32	NA	+++	NA	+++	NA none
9	T <sub>4</sub> NM <sub>2</sub> post radical prostatectomy metastases	3xAd5PSMA	MAB	4.47	NA	+++	NA	-	NA +++NA none

Legend:

-; ++; +++: lack (-) or presence of local tumor metastases, or lymph node engagement

MAB: maximum androgen blockade with Zoladex, Casodex or Flucinome

NA: not available

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The results, summarized in Tables 1 and 2, indicate that the progression of metastatic prostate cancer was retarded or stopped in at least some patients, as can be seen by the reduction in PSA; the a reduction of the size of the prostate gland, as estimated by the CAT scan; and/or the absence or reduction of numbers of lymph node and/or bone metastases. For example, patient 2 had less PSA after the treatment; a significant decrease in the size of his prostate gland; and he did not develop any lymph node or bone metastases, suggesting a positive effect of the treatment. Similarly, patient 3 had a much lower PSA level after the treatment, a significant reduction in the size of his prostate gland, and the absence of development of lymph node metastasis. It is likely that the bone metastasis may have been present, but undetectable, prior to the treatment. Patients 5 and 6 also responded positively to the treatment, as can be seen by a reduction in the PSA level; the significant reduction in the size of the prostate gland; and the absence of any metastases in both patients. These patients were still alive two years after having obtained these data. However, patients 1 and 4, who did not respond to the treatment, died.

**Example 5: Phase I/II safety/dose-escalation clinical study with vectors encoding PSMA**

This example describes a phase I/II safety/dose-escalation clinical study conducted on prostate cancer patients, which showed that administration of a viral and/or plasmid vector encoding the extracellular domain of human PSMA alone, or together with a nucleic acid encoding the costimulatory molecule CD86 and/or rhuGM-CSF to subjects is safe and efficient for treating prostate cancer. The five patients described in Table 1 which were still alive were included in this study.

Since PSMA is a self-antigen, inducing an immune reaction requires the presence of self-reactive T lymphocytes and the breaking of self-tolerance. Here, the breaking of self-tolerance was measured by the development of a delayed type hypersensitivity (DTH) response to an injection of the nucleic acid(s).

Reagents:

The cDNAs and plasmids used in this example are the same as those described in Example 4. Briefly, these were constructed as follows. The cDNA encoding for extracellular portion of the human PSMA (XC-PSMA) was cloned into the pCR2.1 vector

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(Invitrogen, Carlsbad, CA) after amplification by RT-PCR of total mRNA, that was obtained from the human prostate cancer cell line LNCaP (CRL 1740, ATCC). The complete human CD86 cDNA was cloned into the pCR2.1 vector by RT-PCR of total mRNA, isolated from human monocytes of a healthy donor.

5 Both the cDNAs were sub-cloned into a modified mammalian expression vector pcDNA 3.1 (Invitrogen, Carlsbad, CA) after deletion of the neomycin resistance gene. Both the XC PSMA and CD86 plasmids contain the corresponding cDNAs under the regulation of a CMV promoter and a Bovine Growth Hormone polyadenylation signal. The combined PSMA/CD86 plasmid contains both the XC PSMA and CD86 cDNAs, each under a  
10 separate CMV promoter and a polyadenylation signal. Both the PSMA and the CD86 plasmids can be expressed in mammalian cells following transfection.

The plasmid-DNA product specifications included endotoxin content below 0.1 EU per microgram of DNA; lack of detectable amounts of bacterial RNA, genomic DNA or ssDNAs as determined by agarose-gel electrophoresis; less than 10 µg of protein per 1 mg  
15 of plasmid DNA as determined by colorimetric assay (Bio-Rad, Hercules, CA). Prior to injection, the plasmids were diluted with sterile pyrogen-free saline.

The entire expression cassette from the XC PSMA plasmid described above, was inserted in a replication deficient (E1, E3 deletions) adenoviral (Ad5) vector (Quantum Biotechnologies Inc., Toronto, Canada). The resulting adenovirus Ad5-PSMA is a  
20 replication deficient recombinant adenovirus in which the replication-essential genes E1 and E3 are replaced with the expression cassette containing a coding sequence for an extracellular portion of the human PSMA. Prior to injection, the Ad5PSMA was diluted with sterile pyrogen-free saline.

Soluble GM-CSF (sGM-CSF, Leukine, Sargramostim) is a recombinant human  
25 granulocyte-macrophage colony-stimulating factor produced by recombinant DNA technology in a yeast expression system (Immunex, Seattle, WA). For injection GM-CSF was reconstituted and diluted according to manufacturer's guidelines.

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Study design:

All patients signed an informed consent form before admission into the study. Data from monitoring visits were shared with the patients as the study proceeded, and the patients were reminded that they were free to withdraw from participation at any time.

5        A total of 26 patients were included in the study. All patients were immunized intradermally around the naval area. An initial group of sixteen patients received two inoculations of 100 ug plasmid(s) at one-week intervals, with or without 40,000 IU sGM-CSF. All patients who received sGM-CSF were injected with 40,000 IU sGM-CSF at the same site on day 2 following each plasmid application.

10       The first four patients accessed into the study received both the PSMA plasmid and the CD86 plasmid but no sGM-CSF. A second group of six patients was injected with a cocktail of the PSMA plasmid and sGM-CSF. A third group of three patients received the PSMA plasmid, the CD86 plasmid and sGM-CSF at the same inoculation site. Finally, a  
15       fourth group of three patients received a cocktail of the combined PSMA/CD86 plasmid with sGM-CSF.

One week following the second immunization all patients were challenged intradermally with 100 ug of PSMA plasmid around the naval area. The DTH response at the injection site was measured at 6, 12, 24, and 48 hours later.

20       Ten weeks after the initial inoculation, all sixteen patients, and a new group of ten patients, received an intradermal injection with  $5 \times 10^8$  PFU of the recombinant Ad5PSMA. The sixteen prior patients, and seven of the new patients, were additionally immunized twice at weekly intervals with 100 ug of PSMA/CD86, and 40,000 IU GM-CSF. The other three patients from the new group received two more immunizations with  $5 \times 10^8$  PFU of Ad5PSMA, each one-week apart.

25       After an additional 2.5 months all patients were again tested for DTH against the PSMA plasmid. Following that, all patients have been on regular boosts, at 3-week intervals, with either the PSMA/CD86 plasmid and sGM-CSF or with the Ad5PSMA virus, some of them longer than one year.

30       The 24-hour DTH reaction was recorded following each re-immunization, the aim being to maintain an intense response following each application. Depending on the



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intensity of the DTH response patients' doses for the following boost have varied from 100 to 800 ug of DNA. The viral dose, whenever virus was applied, has always been  $5 \times 10^8$  PFUs.

Constant monitoring of the clinical state and the vital signs was carried out for 2  
5 hours after vaccination. If stable, the subject was allowed to leave the hospital. A brief follow-up visit occurred 24 hours (and 48 hours in the case of GM-CSF inoculation) later.

#### Monitoring studies

Standard laboratory tests included CBC, urinalysis, liver enzymes, antinuclear  
antibodies, erythrocyte sedimentation rate, PSA. Each patient had a pelvic CAT scan, chest  
10 radiograph and a cardiograph on entry and on week 20 (week 10 for the 10 patients immunized with virus only). Additionally, analysis of HLA DR<sup>+</sup>; CD4<sup>+</sup>; CD8<sup>+</sup>; CD3<sup>-</sup> /CD16<sup>+</sup>CD56<sup>+</sup>; CD3<sup>+</sup>; CD11b<sup>+</sup>; CD25<sup>+</sup>; CD19<sup>+</sup> cells as well as CD4/CD8 ratio, prior to, and following immunotherapy, were performed by flow cytometry. Safety was defined as  
15 lack of untoward clinical or laboratory events, with particular attention to local and systemic reactions, as well as evidence of anti-nuclear or anti-double-stranded DNA antibody.

#### RESULTS

##### Safety:

All immunizations were well tolerated. No changes in vital signs occurred following  
20 injections or on follow-up visits. No significant changes occurred in erythrocyte sedimentation rate, CBC, serum creatinine or other blood chemistries, or urinalysis. Serum liver chemistry values remained within normal range in all subjects. No significant changes in the analysis of CD/HLA DR<sup>+</sup>; CD4<sup>+</sup>; CD8<sup>+</sup>; CD3<sup>-</sup>/CD16<sup>+</sup>CD56<sup>+</sup>; CD3<sup>+</sup>; CD11b<sup>+</sup>; CD25<sup>+</sup>; CD19<sup>+</sup> cells as well as CD4/CD8 ratio, prior to, and following immunotherapy  
25 were detected. No subject developed abnormal vital signs following injection, no significant increases in antinuclear antibody titer were observed, and no anti-double-stranded DNA antibodies were detected. One patient had a papular urticaria-like rash, with small petechiae at the center which developed 24 hrs after the last plasmid immunization. This rash disappeared after discontinuation of the antibiotic therapy that he was receiving  
30 for an unrelated condition. One patient had a vesicular rash after the third viral

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immunization. The rash was located on the back, and it resolved in the next two days with no treatment.

#### Immunization rate

A normal healthy volunteer, together with two patients received intradermally, at two separate sites, 100 ug of empty pcDNA3.1 vector and 40,000 IU of soluble GM-CSF. There was no DTH-like reaction at either of the application sites 24 and 48 hours after the injection (fig.2). Similarly, the first application of PSMA or PSMA/CD86 plasmids, with or without GM-CSF, in the other patients resulted in no reaction.

In contrast, in some patients, the second, and especially the third PSMA or PSMA/CD86 plasmid application, led to a measurable in vivo delayed type hypersensitivity response with an infiltrate that peaked in 48 hours and in some instances exceeded 40 mm (fig.2). Using this DTH-like response, patients who were immunized against PSMA could be identified.

All patients who received sGM-CSF together with both the PSMA and the CD86 plasmids, or the complex PSMA/CD86 plasmid, developed positive DTH following the third plasmid application (fig.3, groups 3 and 4). In contrast, only two of the four patients who received the plasmids without sGM-CSF, and four of the six patients immunized without the CD86 plasmid, developed positive DTH following the third plasmid application.

In addition, all 10 patients who received an initial immunization with the Ad5PSMA became immunized and developed positive DTH following PSMA plasmid inoculation. Of the four patients who did not immunize in the first series (fig.3, groups 1 and 2), all became immunized following immunizations with Ad5PSMA.

#### Effect of therapy

This was a phase I/II safety-dose escalation study. The relatively small patient population varied widely with respect to their disease status and prior or concurrent treatment so that extreme caution must be exercised in judging the effectiveness of the vaccine therapy, particularly in patients receiving concurrent hormone therapy (GM-CSF therapy). Disease improvement, when present, was implied by local tumor regression, fall

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in PSA in patients not receiving hormone therapy, and decrease in bone pains when bone metastases were present. The twenty-six patients can be divided into two main groups:

**I. Patients who had prior radical prostatectomy:**

Six patients had had radical prostatectomy before entering the study.

5 (i) Patients with biochemical recurrence and no detectable lymph node or bone metastases. Three patients presented with biochemical recurrence (rising PSA values) 1.5 to 3.5 years following radical prostatectomy. They were all treated solely by immunotherapy. PSA values in all three of them have stabilized between 1 and 2 ng/ml following the onset of immunization (fig.4.), although it is not rare to observe such evolutions in untreated  
10 patients. Currently they have no complaints and are considered to have a "stable" disease. A representative case is shown in fig.4.

(ii) Patients with detectable metastases. The other three patients had detectable metastatic disease – one with distant LN involvement and two with bone metastases. They were all installed on combined hormone and immune therapy. Two of the patients were not  
15 influenced by the therapy. The third patient had a PSA of 30 ng/ml and severe bone pains. He was on combined hormone and immune therapy for four months and had been only on immunotherapy for the last seven months. His PSA has been maintained at 6 ng/ml and he has no bone pain or local disease symptomology.

20 **II. Patients with no prior radical prostatectomy:**

Twenty patients with no prior radical prostatectomy were admitted into the study:

II.1. Patients who underwent radical prostatectomy following combined hormone and immune therapy: Four of the 20 patients had advanced local disease and received adjuvant immuno- and hormone therapy prior to surgery. DRE performed a day before  
25 surgery on three of them who had been on therapy longer than three months, revealed no palpable gland. On surgery the gland appeared significantly shrunken. The urologist also noted in these three cases an increased number of newly formed blood vessels lying at the fibromuscular layer. These vessels had walls that were easily injured and had increased tendency for bleeding. All coagulation tests in these patients were otherwise normal. All  
30 four patients remained on immunotherapy following prostatectomy. Three of them,

however, have now (3-6 months following surgery) evidence of biochemical recurrence. A representative case from this group is shown on fig.5.

II.2. Patients with no radical prostatectomy or bone metastases: Nine patients had advanced local disease but no evidence of metastatic disease.

5 (i) Patients on combined hormone and immune therapy: Seven patients, ages 63 to 74 from this group were on combined hormone and immunotherapy. They all improved following treatment. One patient who had significant improvement locally (prostate gland shrinkage, no obstructive voiding symptoms) was removed from hormone therapy at 10 months (see figure 6), and remained on immunotherapy only. Currently, five months  
10 following discontinuation of hormone therapy, he has no evidence of local recurrence or metastatic disease (fig.6).

(ii) Patients on immunotherapy only: The two patients in this group were on immunotherapy only. The first patient was not influenced by the immunotherapy, had evidence of local disease progression and was recently started also on hormone therapy.  
15 The second patient, however, responded to the therapy with tumor shrinkage and drop in PSA from 13 to 4.5 ng/ml and is considered a responder (fig.7).

II.3. Patients who had not undergone radical prostatectomy and who had metastatic disease: The last seven patients had metastatic disease, and no prior surgery. One had  
20 distant lymph node involvement, and 6 had bone metastases.

(i) Patients on combined hormone and immune therapy. Four patients were on combined hormone and immune therapy. One patient (T4bNxM1b) did not respond to therapy. The other three had marked local improvement, decrease in bone pain (for those with bone metastases) and fall in PSA, although the concurrent hormone therapy renders  
25 these data difficult to interpret. However, the patient with LN involvement has been off hormone therapy for the last seven months with no signs of disease recurrence (fig.8).

(ii) Patients on immunotherapy only: Three patients with bone metastases refused hormone therapy and were left on immunotherapy only. One of them is in stable condition, with PSA between 10 -12, the bone pain has diminished, the local status has

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improved and he currently has no obstructive voiding symptoms. The other two patients were not influenced by the therapy and are considered non-responders.

In summary, 26 patients were included in the study, ten with, and 16 without, detectable metastases (Table 3). Fifteen patients improved following the therapy, 6 of them being solely on immunotherapy.

Table 3. Effect of combined hormone and immune therapy, or immune therapy only, on patients with prostate cancer.

Patients with detectable LN or bone metastases				Patients with no detectable metastases			
Combined hormone and immune therapy		Immune therapy only		Combined hormone and immune therapy		Immune therapy only	
Responder	Non-responder	Responder	Non-responder	Responder	Non-responder	Responder	Non-responder
2	5	1	2	7	0	5	4

Thus, in this phase I/II safety-dose escalation study, all patients became immunized against PSMA as demonstrated by the development of positive DTH reaction, although the extent of their response depended on the advancement of their disease, such as the presence of distant metastase. Briefly, twelve of the sixteen patients who had no evidence of distant metastases, including five which were treated by immunotherapy only, responded to the therapy with a drop in PSA levels and, whenever applicable, with improvement of local disease. In contrast, only three of the ten patients with detectable distant metastases showed any improvement. It is possible that a large tumor load may have an anergizing effect on immune T cells [25,32] or that class I expression was decreased or lost in cancer cells. Downregulation or loss of HLA on tumor cells in metastases may be the reason that 7 of the 10 patients with metastatic disease in this study did not improve following treatment (table 1).

These clinical trials have shown that evoking of a T-cell immune response against PSMA does not lead to autoimmune reactions in other organs. This study also demonstrates that repeated intradermal injection of rHuGM-CSF (Sargramostim) is a safe

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procedure and that in vivo transfection with CD86 is also well tolerated. In addition, soluble recombinant GM-CSF, as well as co-expression of CD86 with the target antigen increases the likelihood for successful immunization. Use of a cocktail of a complex PSMA/CD86 plasmid and sGM-CSF leads to uniform immunizations in all patients. Thus, a preferred method for induction of anti-PSMA T cell immunity includes administration of a PSMA/CD86 plasmid and soluble GM-CSF.

This is one of the first report on DNA vaccination in humans, and probably the first one that involves patients who were repeatedly immunized for more than one year. The primary objective of the study was to determine the safety of the PSMA vaccine after repeated intradermal injections. So far, almost one and a half years since the study began, no patient has experienced any short- or long-term side effects, including anti-DNA antibody. The only exception is the development of fragile vasculature in the immediate vicinity of the prostate in three of the four patients who had immunotherapy prior to surgery.

In conclusion this example shows that: (1) repetitive DNA and recombinant adenoviral immunizations of humans are a safe procedure; (2) tolerance to self-antigens can be broken by immunizations with DNA that encodes the antigen, inserted in either a plasmid or in a recombinant viral vector; and (3) addition of DNA encoding co-stimulatory molecules and soluble GM-CSF increases the immunization rate to 100%.

#### **Example 6: Phase II study of treatment of prostate cancer patients with vectors encoding PSMA**

##### Study design:

All vaccines were inoculated intradermally taking caution that all the infusate is retained as a blister at the epidermal-dermal junction. The patients were immunized intradermally around the naval area. Constant monitoring of the clinical state and the vital signs was carried out for 2 hours after vaccination. If stable, the subject was allowed to leave the hospital. A brief follow-up visit occurred 24 (48 in the case of granulocyte-macrophage colony-stimulating factor (GM-CSF) inoculation) hours later.

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Sixteen prostate cancer patients received three intradermal plasmid inoculations of 100 µg at one-week interval with or without 40,000 IU sGM-CSF (Leukine, Immunex, Seattle). All patients were monitored for side effects for 2.5 months. These patients and a new group of 10 patients then received three intradermal injections at one-week intervals with  $5 \times 10^8$  PFU of the recombinant Ad5PSMA. Patients were monitored for side effects for 2.5 months and then tested for DTH against a PSMA plasmid challenge. These patients have been on regular boosts with the PSMA plasmid, the PSMA/CD86 plasmid, or the Ad5PSMA viral vector at 3-week intervals up to one year after the start of the study. After that all patients have been on regular boosts at 3-month intervals alternating between PSMA plasmid and Ad5PSMA.

A phase II study to evaluate the biological effectiveness of immunotherapy was initiated 8 months after the beginning of the first clinical trial study. Patients were initially immunized twice, 3 weeks apart, with a cocktail of 200 µg PSMA/CD86 plasmid and 80,000 IU sGM-CSF. 40,000 IU sGM-CSF was injected at the immunization site 6 and 24 hours later. An intradermal boost with  $5 \times 10^8$  PFUs Ad5PSMA was given 3 weeks later. Booster immunizations are currently alternated between a cocktail of 100 µg PSMA plasmid plus 40,000 sGM-CSF and  $5 \times 10^8$  PFUs Ad5PSMA at 3-month intervals.

### Monitoring

Standard laboratory tests included CBC, urinalysis, liver enzymes, anti-DNA antibodies, erythrocyte sedimentation rate, PSA. Each patient had a CAT scan, chest radiograph and a cardiograph on entry and on week 20 (week 10 for the 2 patients immunized with virus only). Patients with prostate cancer had nucleotide bone scans at the time of admission into the study and at 6 months (metastatic disease) or 12 months-intervals. Analysis of CD/HLA DR<sup>+</sup>; CD4<sup>+</sup>; CD8<sup>+</sup>; CD3<sup>+</sup>/CD16<sup>+</sup>CD56<sup>+</sup>; CD3<sup>+</sup>; CD1<sup>+</sup>; CD25<sup>+</sup>; CD19<sup>+</sup> cells, IL-4<sup>+</sup> and γ IFN<sup>+</sup> T cells, as well as CD4/CD8 ratio prior to and following immunotherapy was performed by flow cytometry. For intracellular lymphokine measurements, patient's whole peripheral blood were stimulated with PMA and ionomycine for 4 hours at 37°C in 5% CO<sub>2</sub> and cytokine exclusion was blocked by Brefeldin A. For anti-PSMA antibodies patients LNCaP cells were incubated with patients sera, washed and stained with F(ab')<sub>2</sub> anti-human IgG and IgM.

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For immunoblotting, after PAA gel electrophoresis in 14% gels, LNCaP cell extracts were electroblotted at 20 V overnight on nitrocellulose membranes in tris-glycine buffer with 20% methanol. Blots were blocked with 3% skimmed milk for 1 hour and incubated for an additional hour with either patients serum (dilution 1:1000) or with a concentrated supernatant from hybridoma HB 11430 (control, dilution 1:1000). After three washes (each for 5 minutes) in 3% skimmed milk-0.05% Tween-20, the blots were incubated for 1 hour with peroxidase-conjugated goat anti-human antibody (experimental) or goat anti-mouse antibody (both in dilution 1:3000). Following 3 washes with 3% milk-0.05 Tween-20 the blots were developed with ECL substrate.

- 10 Safety is defined as lack of untoward clinical or laboratory events, with particular attention to local and systemic reactions as well evidence of anti-DNA antibody.

#### Statistical analysis

The difference between the results from different sub-groups of patients and healthy subjects was tested with the Student t-test. The level of significance was set at  $p < 0.05$ .

## 15 RESULTS

- All immunizations were tolerated well. No changes in vital signs occurred following injections or on follow-up visits. No significant changes occurred in erythrocyte sedimentation rate, CBC, serum creatinine or other blood chemistries, or urinalysis. Serum liver chemistry values remained within normal range in all subjects. No subject developed abnormal vital signs following injection and no antinuclear or anti-DNA antibodies were detected.

- All patients became immunized against PSMA as shown by the development of a DTH reaction at the site of the PSMA plasmid application 24 and 48 hours after inoculation. None of the patients developed antibodies to PSMA as tested by flow cytometry or Western blotting. The likelihood of becoming immunized in prostate cancer patients increased with the use of the complex (PSMA/CD86) plasmid and/or soluble GM-CSF. All prostate patients who did not immunize to PSMA in the first series of experiments (immunization solely with the PSMA plasmid) became so following immunizations with the Ad5PSMA vector.



### Changes in peripheral lymphocyte subsets

The prostate cancer patients group is extremely heterogeneous with respect to the stage and the progression of the of disease, as well as to the type of treatment received. The overall analysis of the subtypes of peripheral blood mononuclear cells in all prostate cancer patients prior to, and following 15 months of continuous immunotherapy, revealed no significant changes:

Table 5. Subsets of peripheral blood lymphomononuclear cells and intracellular lymphokines in prostate cancer patients prior to and following 15 months of immunotherapy (Each value represents mean value  $\pm$  SD of % of mononuclear cells).

Lymphocyte subsets	Prior to IT (% cells) n=36	After 15 mos of continuous IT (% cells) n=32	Student T test
CD3+	64.02 $\pm$ 13.58 n=36	62.73 $\pm$ 13.01 n=32	
CD4+	39.67 $\pm$ 8.11 n=32	36.19 $\pm$ 7.79 n=32	
CD8+	38.57 $\pm$ 9.1 n=36	35.625 $\pm$ 6.5 n=32	
CD3+/CD16+56+	26.16 $\pm$ 13.41 n=36	26.59 $\pm$ 13.24 n=32	
CD4/CD8 ratio	1.136 $\pm$ 0.45 n=36	1.059 $\pm$ 0.31 n=32	
CD19+	9.52 $\pm$ 3.84 n=36	10.09 $\pm$ 3.86 n=32	
CD25+	32.54 $\pm$ 9.37 n=35	27.0 $\pm$ 7.57 n=32	Two tail p=0.010
CD11b+	49.91 $\pm$ 9.86 n=36	52.72 $\pm$ 8.7 n=32	
CD3+/DR+	11.03 $\pm$ 5.4 n=36	8.53 $\pm$ 4.47 n=32	Two tail p=0.043
IL-4+ T cells	2.11 $\pm$ 1.76 n=18	3.78 $\pm$ 2.19 n=35 / 10 months of IT	Two tail p=0.026
IL-4+ T cells	2.11 $\pm$ 1.76 n=18	3.77 $\pm$ 2.19 n=21	Two tail p = 0.0141
IFN+ T cells	32.65 $\pm$ 11.8 n=18	40.39 $\pm$ 14.02 n=35/10 months of IT	Two tail p=0.057
$\gamma$ IFN+ T cells	32.65 $\pm$ 11.8 n=18	40.33 $\pm$ 12.56 n=21	Two tail p=0.0649

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The percentage of circulating NK cells decreases during the first 5 months of therapy but rebounds to initial value during the next 5 months and does not change later. The percentage of CD4 T cells, as well as and CD4/CD8 ratio, has a tendency to decrease and they are lower for patients with metastases. The activation markers on T cells (DR and CD25) show a tendency to decrease following therapy, while CD11b+ T cells remain relatively constant. Cancer patients have higher numbers of  $\gamma$ IFN+ and IL-4+ T cells than healthy subjects. The percentage of T cells expressing  $\gamma$ IFN has a tendency to increase during therapy as does the percentage of IL-4+ T cells. The increase of IL-4+ T-cells seems to be delayed in time and attenuated in prostatectomized patients with biochemical recurrence. The observed tendencies do not reflect the normal history of the disease since we have observed them only in patients treated by immunizations.

#### Effectiveness of therapy

The results show that immunotherapy is clinically effective for selected patients. The success of the treatment clearly depends on the stage of the disease, being mostly effective in prostatectomized patients with biochemical recurrence. In nine of the 13 patients who had rising PSA following prostatectomy, PSA became undetectable following 3-4 immunizations and in 1 patient PSA remained flat (table 5). Most of the patients with a large tumor load (distant metastases) did not improve following immunotherapy and required additional hormone therapy. Some patients with advanced local disease improved following immunotherapy with tumor shrinkage and fall in PSA (Table 6).

Table 6. Results from a phase I/II study on prostate cancer treatment by IT and combined therapy.

Type of therapy	Immunotherapy only				Combined immune and hormone therapy			
Type of response	R	PR	NR	Total	R	PR	NR	Total
Type of patients								
Patients with advanced local disease	1	1	6	7	9	0	3	12
Patients with distant metastases	1	1	1	3	6	3	9	18
Postprostatectomy patients with rising PSA	9	1	3	13	1			1
Grand Total	11	3	9	23	16	3	12	31

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On this table, as well as everywhere else in the text, a response – R – is defined as a fall in PSA and, where applicable, reduction in tumor volume and in symptomatology such as obstructive voiding and bone pains. A partial response (PR) is defined as no increase in PSA, no tumor growth, no obstructive symptomatology and decrease in bone pains where applicable. A lack of response (NR) is defined as progression of disease, rise in PSA and increase in tumor volume leading to obstructive voiding symptoms and/or bone pains where applicable.

#### Flow Cytometry Changes and Effectiveness of Immunotherapy

In an attempt to correlate changes in the flow cytometry parameters with effectiveness of therapy, we analyzed flow cytometry phenotypes of responders and non-responders to IT. The next two figures summarize flow cytometry data prior to and after 15 months of continuous IT (figs.9 and 10). Repetitive naked DNA and adenoviral immunizations lead to statistically significant changes in the percentage of their T cells that express  $\gamma$ IFN when patients are divided in subgroups based on their response to therapy.

As shown in Figure 10, the patients who respond to therapy, including sole immunotherapy, tend to have higher percentage of CD3+, CD25+ and CD4+ T cells and lower numbers of CD3-CD56+ NK cells prior to initiation of treatment relative to patients who do not respond. The percentage of CD11b+ cells in peripheral blood increases in those patients who do not respond to therapy, including sole immunotherapy. CD25+ and DR+ T cells decrease in those patients who do not respond to IT and remains relatively unchanged in responders. IL-4+ T cells and  $\gamma$ -IFN+ T cells increase following IT and the increase is higher in patients who respond to immunotherapy. These changes are observed also in colorectal cancer patients who do not receive adenoviral immunizations and should not therefore be attributed solely to the use of the viral vector.

A “healthy” phenotype that will most-likely lead to improvement of patient’s disease following immunotherapy, therefore, involves high percentage of CD3+ and CD4+ cells and low percentage of NK cells and DR+ T cells in peripheral blood. Interestingly, conversion to such a phenotype in patients with advanced colorectal cancer is achieved

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following chemotherapy and this raises important questions of when and following what treatment should IT be applied (fig.11).

Thus, the results of this study show that nine of the 12 patients with advanced local prostate cancer responded to combined hormone and immune treatment. Of the 18 patients with distant bone metastases 6 responded with reduction in bone pain and at least 50% fall in PSA.

This study further shows that DNA immunization leads to an increase in the percentage of  $\gamma$ IFN producing T cells, and that this correlates with clinical improvement. There is a significant increase, however, in the percentage of IL-4-producing T cells, which may be mediated by the CD86-containing plasmid. Experimental work in animals has shown that the intensity of the immune response can be increased by the introduction of sequences encoding for costimulatory molecules (CD86) (Moro et al. Cancer Res 1999; 59:2650-2656) or soluble lymphokines (GM-CSF) (Armitage JO Blood 1998;92:4491-4508), but their use seems to lead to a mixed Th0-type of response. Overall, this may not be so disadvantageous since induction of autoimmunity to treat cancer may require the simultaneous induction of Th1 and Th2 responses (Hung et al. J Exp Med. 1998 Dec 21;188:2357-68). Cytokines produced by these CD4+T cells activate eosinophils as well as macrophages, which then collaborate within the site of tumor challenge to cause its destruction (Hung et al., supra). In support of this, in prostatectomized patients who are solely on immunotherapy (IT), fall in PSA correlates with an increase in IL-4+ T cells.

The flow cytometry results described above indicated that, compared to healthy controls, patients with advanced disease have lower CD3+ and CD4+ T cells and higher CD8+ T and NK cells. Such a phenotype correlates with poor prognosis for immunotherapy but can be reversed towards a "healthier" phenotype after chemotherapy. This may be explained as follows: in a conventional immune response dendritic cells, which reside in tissues, become alerted by some form of tissue damage or pathogen products, migrate to the regional lymph node and stimulate antigen-specific T cells. The type of interaction between the dendritic cells and T cells is critical for the outcome of the immune response. Both T cells and dendritic cells mature and change phenotype during this interaction and, at the end, the T cells induce the dendritic cells into programmed cell death (apoptosis) (Garside et al. Science 1998;281:96-99 and De Smedt et al. J Immunol 1998 16:4476).

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Dendritic cells reside in healthy as well as in cancer tissues. Cancer cells bear tumor-associated antigens that can serve as targets for immune recognition and attack. Tumor cells, however, secrete numerous lymphokines, such as TGF-beta, IL-10 and PGE-2, that inhibit dendritic cell maturation and function (Kiertscher et al. J Immunol 2000;164:1269). Such "incompetent" DCs may transport tumor-associated antigens to the regional lymph nodes but they fail to stimulate T cells into potent effector cells capable of rejecting tumor. As a result the antigen persists, ineffective antigen presentation continues until the system is finally exhausted. At that time disease prevails and rapidly progresses. This "vicious" circle may be overcome early in disease by loading "healthier" DCs with antigen at sites distant to the immunosuppressive tumor milieu. In advanced disease, however, such a maneuver is insufficient. When this point is reached, immune rejuvenation is necessary. Chemotherapy seems to be an obvious option because it interrupts the cycle. Choice of drugs, as well as dose and schedule of administration will probably have a major impact (Nigam et al. Int J Oncol 1998 12:161).

**Example 7: Phase I clinical trial on colorectal cancer using naked DNA immunization against CEA**

The plasmid encoding the human CEA is identical to that encoding PSMA described above, except that a sequence encoding the full length human CEA devoid of signal sequence was included, instead of PSMA. No CD86 was included in this plasmid. Following transfection of DCs with the CEA vector, DCs express peptides derived from the CEA protein in association with MHC class I and class II molecules and stimulate a CD4 and CD8 cytotoxic T cell response against colorectal cancer cells.

The patients were immunized at the volar side of the forearm. All vaccines were inoculated intradermally taking caution that all the infusate is retained as a blister at the epidermal-dermal juncture. Since immunization with the CEA plasmid often led to swelling of the axillary lymph nodes, the arm opposite to the metastatic side was used when liver or lung metastases were present. Constant monitoring of the clinical state and the vital signs was carried out for 2 hours after vaccination. If stable, the subject was allowed to leave the hospital. A brief follow-up visit occurred 24 (48 in the case of granulocyte-macrophage colony-stimulating factor (GM-CSF) inoculation) hours later.

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All patients were initially immunized intradermally three times, 3 weeks apart, with a cocktail of 200 µg CEA plasmid and 80,000 IU sGM-CSF. 40,000 IU sGM-CSF was injected at the immunization site 6 and 24 hours later. Booster immunizations with a cocktail of 200 µg CEA plasmid plus 40,000 sGM-CSF are currently given at 3-month intervals.

Monitoring the immune response and condition of the subjects was performed as described in Example 5. The statistical significance was also determined as described in Example 5.

All immunizations were tolerated well. No changes in vital signs occurred following injections or on follow-up visits. No significant changes occurred in erythrocyte sedimentation rate, CBC, serum creatinine or other blood chemistries, or urinalysis. Serum liver chemistry values remained within normal range in all subjects. No subject developed abnormal vital signs following injection and no antinuclear or anti-DNA antibodies were detected.

All patients became immunized against CEA as shown by the development of a DTH reaction at the site of the CEA plasmid application 24 and 48 hours after inoculation.

### **Equivalents**

It will be apparent to those skilled in the art that the examples and embodiments described herein are by way of illustration and not of limitation, and that other examples may be used without departing from the spirit and scope of the present invention, as set forth in the claims.

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**Claims:**

1. A method for inducing a cell-mediated immune response against a cell comprising a target antigen in a subject, comprising administering to the subject  
5 a polynucleotide encoding a variant of the target antigen, such that the polynucleotide is expressed in a cell and then a cell-mediated immune response is induced against cells carrying the target antigen; wherein the variant remains in the cytosol following its expression and is completely processed for major histocompatibility complex (MHC) presentation.
- 10 2. The method of claim 1, wherein the variant is biologically inactive.
3. The method of claim 1, wherein the antigen is a tumor antigen.
4. The method of claim 1, wherein the antigen is an auto-antigen.
5. The method of claim 1, wherein the antigen is a heterologous antigen.
6. The method of claim 5, wherein the antigen is a viral antigen.
- 15 7. The method of claim 3, wherein the antigen is a prostate specific antigen.
8. The method of claim 7, wherein the antigen is prostate specific membrane antigen (PSMA).
9. The method of claim 8, wherein the variant of the target antigen comprises the extracellular domain of PSMA, or a portion thereof sufficient to be presented by  
20 MHC class I complexes.
10. The method of claim 9, wherein the variant of the target antigen comprises amino acids 44-750; 55-750; 61-750; 76-750; or 94-750 of SEQ ID NO: 2.
- 25 11. A method for reducing the number of undesired cells in a subject, comprising administering to the subject a pharmaceutically efficient amount of a polynucleotide encoding a variant of a target antigen of the undesired cells operably linked to a transcriptional control sequence, such that the polynucleotide is expressed in a cell and then the number of undesired cells is

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reduced in the subject; wherein the variant remains in the cytosol following its expression and is completely processed for MHC presentation.

12. The method of claim 11, wherein the variant is biologically inactive.
13. The method of claim 11, wherein the undesired cells are tumor cells and the target antigen is a tumor antigen.
14. The method of claim 13, wherein the target antigen is PSMA, PSA or PAP.
15. The method of claim 14, wherein the variant of the target antigen comprises amino acids 44-750; 55-750; 61-750; 76-750; or 94-750 of SEQ ID NO: 2 or SEQ ID NO: 4.
16. The method of claim 13, wherein the target antigen is a MAGE antigen or MART-1.
17. The method of claim 16, wherein the variant of the target antigen comprises the amino acid sequence set forth in SEQ ID NO: 10, 12, or 14.
18. The method of claim 13, wherein the target antigen is CEA.
19. The method of claim 18, wherein the variant of the target antigen comprises the amino acid sequence set forth in SEQ ID NO: 6.
20. The method of claim 13, wherein the target antigen is Her2/Neu.
21. The method of claim 20, wherein the variant of the target antigen comprises the amino acid sequence set forth in SEQ ID NO: 8.
22. The method of claim 13, wherein the polynucleotide encoding a variant of the target antigen is included in a viral vector.
23. The method of claim 22, wherein the viral vector is an adenovirus vector.
24. The method of claim 23, wherein the undesired cells are prostate cancer cells and the viral vector is a replication-defective recombinant adenovirus comprising a nucleotide sequence encoding amino acids 44-750; 55-750; 61-750; 76-750; or 94-750 of SEQ ID NO: 2.
25. The method of claim 24, wherein the viral vector is Ad5-PSMA, having ATCC Designation Number VR 2362.



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26. The method of claim 12, wherein the undesired cell is a cell infected with an infectious agent and the target antigen is an antigen from the infectious agent.
27. The method of claim 26, wherein the infectious agent is a virus and the target antigen is a viral protein.
- 5 28. A method for treating a subject having undesired cells, comprising (i) obtaining professional antigen presenting cells from the subject; (ii) contacting said cells *ex vivo* with a polynucleotide encoding a variant of a target antigen of the undesired cells operably linked to a transcriptional control sequence, which variant remains in the cytosol and is completely processed for MHC presentation; and (iii) administering the cells obtained in (ii) to the subject; such that the number of undesired cells is reduced in the subject.
- 10 29. The method of claim 28, wherein the variant is biologically inactive.
30. A plasmid vector encoding a variant of human PSMA, wherein the variant remains in the cytosol following its expression in a cell and is completely processed for MHC presentation; and is biologically inactive.
- 15 31. The plasmid vector of claim 30, wherein the variant comprises a nucleotide sequence a nucleotide sequence encoding amino acids 44-750; 55-750; 61-750; 76-750; or 94-750 of SEQ ID NO: 2.
32. The plasmid vector of claim 31, having ATCC Designation 203168.
- 20 33. A viral vector encoding a variant of human PSMA, wherein the variant remains in the cytosol following its expression in a cell and is completely processed for MHC presentation; and is biologically inactive.
34. The viral vector of claim 33, wherein the variant comprises a nucleotide sequence a nucleotide sequence encoding amino acids 44-750; 55-750; 61-750; 76-750; or 94-750 of SEQ ID NO: 2.
- 25 35. The viral vector of claim 34, which is an adenoviral vector.
36. The viral vector of claim 35, having ATCC Deposit Number VR 2361.
37. The method of claim 1, wherein the antigen is an oncogene or a proto-oncogene.

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- 5 38. A method for stimulating antigen presentation of a target antigen by a cell, comprising introducing into the cell a nucleic acid encoding a variant of a target antigen operably linked to a transcriptional control sequence, such that the variant is expressed in the cell and antigen presentation of the antigen by the cell is induced, wherein the variant remains in the cytosol following its expression and is completely processed for MHC presentation.
39. The method of claim 38, wherein the variant is targeted to intracellular degradation pathways in the cytosol.
40. The method of claim 38, wherein the variant is ubiquitinated.
- 10 41. The method of claim 38, wherein the variant is degraded into peptides.
42. The method of claim 41, wherein at least some of the peptides are presented on the surface of the cell within MHC complexes.
43. The method of claim 38, wherein the cell is a professional antigen presenting cell.
- 15 44. The method of claim 1, wherein the variant is a fragment of the antigen.
45. The method of claim 44, wherein the antigen is a cell membrane protein comprising a transmembrane domain, and the variant is a form of the cell membrane protein which does not comprise a functional transmembrane domain.
- 20 46. The method of claim 45, wherein the transmembrane domain comprises a deletion.
47. The method of claim 46, wherein the transmembrane domain is deleted.
48. The method of claim 45, wherein the transmembrane protein comprises a cytoplasmic domain and the variant does not comprise a functional cytoplasmic domain.
- 25 49. The method of claim 48, wherein the cytoplasmic domain comprises a deletion.
50. The method of claim 49, wherein the cytoplasmic domain is deleted.

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51. The method of claim 44, wherein the antigen is a secreted protein and the polynucleotide encoding the variant antigen lacks a functional signal peptide sequence.
52. The method of claim 51, wherein the signal peptide sequence comprises a deletion.
53. The method of claim 52, wherein the signal peptide sequence is deleted.
54. The method of claim 44, wherein the antigen is a nuclear protein and the variant antigen lacks a functional nuclear translocation signal.
55. The method of claim 38, wherein the variant further comprises a modification that renders the variant biologically inactive.
56. The method of claim 38, wherein the antigen is PSMA.
57. The method of claim 48, wherein the antigen is PSMA.
58. The method of claim 51, wherein the antigen is PSMA.
59. The method of claim 58, wherein the transmembrane and cytoplasmic domains are deleted.
60. The method of claim 58, wherein the variant comprises a portion of the extracellular domain of PSMA sufficient for presentation by an MHC complex.
61. The method of claim 60, wherein the variant comprises amino acids 44-750; 55-750; 61-750; or 94-750 of SEQ ID NO: 2.
62. The method of claim 61, wherein the variant comprises amino acids 44-750 of SEQ ID NO: 2.
63. A cell presenting fragments of a target antigen in the context of an MHC complex, wherein the cell comprises a nucleic acid encoding a variant of the target antigen operably linked to a transcriptional control sequence, which variant remains in the cytosol following its expression and is completely processed for MHC presentation.
64. The cell of claim 63, wherein the cell is a professional antigen presenting cell.

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65. The cell of claim 64, wherein the antigen is a cell membrane protein comprising a transmembrane domain, and the variant comprises a deletion in the transmembrane domain rendering it functionally inactive.
- 5 66. The cell of claim 64, wherein the antigen is a secreted protein comprising a signal peptide, and the variant comprises a deletion in the signal peptide rendering it functionally inactive.
67. The cell of claim 65, wherein the antigen is PSMA and the variant comprises amino acids 44-750 of SEQ ID NO: 2.
- 10 68. The method of claim 11, further comprising administering to the subject a polynucleotide encoding human CD86 and/or human CD80 operably linked to a transcriptional control sequence.
- 15 69. The method of claim 68, comprising (i) administering to the subject a polynucleotide encoding a variant of a target antigen of the undesired cells operably linked to a transcriptional control element, which variant remains in the cytosol following its expression and is completely processed for MHC presentation, and a polynucleotide encoding human CD86 operably linked to a transcriptional control element; and (ii) administering to the subject a polynucleotide encoding a variant of a target antigen of the undesired cells operably linked to a transcriptional control element, which variant remains in the cytosol following its expression and is completely processed for MHC presentation, and a polynucleotide encoding human CD80 operably linked to a transcriptional control element.
- 20 70. The method of claim 69, wherein step (ii) is performed from about 3 to 42 days after step (i).
- 25 71. The method of claim 69, wherein the polynucleotides of step (i) are administered from one to four times to the subject prior to administering the polynucleotides of step (ii).
72. The method of claim 11, further comprising administering granulocyte macrophage colony stimulating factor (GM-CSF) to the subject.

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73. The method of claim 72, wherein the GM-CSF and the polynucleotide are administered simultaneously to the subject.
74. The method of claim 72, wherein the GM-CSF is administered at a concentration of at about 0.1 to 200  $\mu\text{g}/\text{m}^2$  body surface area.
- 5 75. The method of claim 11, further comprising administering to the subject a polynucleotide encoding human GM-CSF operably linked to a transcriptional control sequence.
76. The method of claim 68, wherein the antigen is PSMA.
77. The method of claim 72, wherein the antigen is PSMA.
- 10 78. The method of claim 68, wherein the antigen is PSA or PAP.
79. The method of claim 72, wherein the antigen is PSA or PAP.
80. The method of claim 76, wherein the undesirable cells are prostate cancer cells.
81. The method of claim 77, wherein the undesirable cells are prostate cancer cells.
82. The method of claim 78, wherein the undesirable cells are prostate cancer cells.
- 15 83. The method of claim 79, wherein the undesirable cells are prostate cancer cells.
84. The method of claim 11, wherein the method comprises administering one or more polynucleotides encoding at least two antigens selected from the group consisting of PSMA, PSA and PAP.
- 20 85. The method of claim 84, wherein a polynucleotide encoding an antigen selected from the group of PSMA, PSA and PAP is administered to the subject from about two to about 42 days after the administration of a nucleic acid encoding an antigen selected from the group of PSMA, PSA and PAP.
86. The method of claim 11, wherein the variant is an allogeneic or xenogeneic form of the antigen.
- 25 87. The method of claim 1, further comprising administering to the subject a pharmaceutically efficient amount of a second polynucleotide encoding a variant of a target antigen of the undesired cells operably linked to a transcriptional control sequence, such that the polynucleotide is expressed in a cell, and wherein

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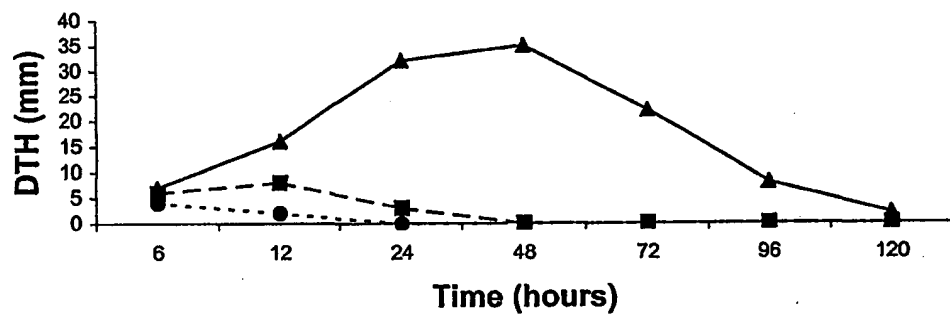
the variant remains in the cytosol following its expression and is completely processed for MHC presentation.

88. The method of claim 87, wherein one polynucleotide is a plasmid and one polynucleotide is a viral vector.
- 5 89. A method for preparing a pharmaceutical composition for treating cancer, comprising combining a plasmid vector of claim 30 and a pharmaceutically acceptable excipient, thereby forming a pharmaceutical composition for treating cancer.
- 10 90. A method for preparing a pharmaceutical composition for treating cancer, comprising combining a plasmid vector of claim 33 and a pharmaceutically acceptable excipient, thereby forming a pharmaceutical composition for treating cancer.

1 mwnllhetds avatarprw lcagallvag gffllglflg wflkssneat nitpkhnmka  
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 241 sypdgwnlpg ggvqrgniln lngagdpltp gypaneyayr rgiaaavglp sipvhpigyy  
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 (SEQ ID NO:2)

Figure 1

Figure 2





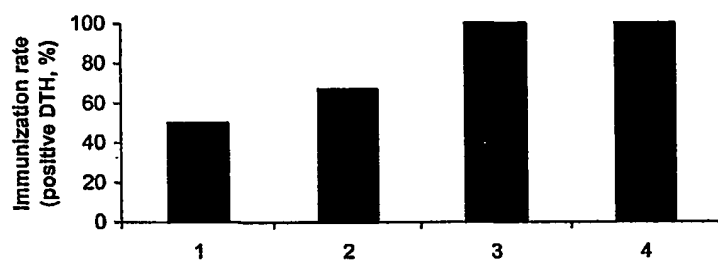
**Figure 3**

Figure 4

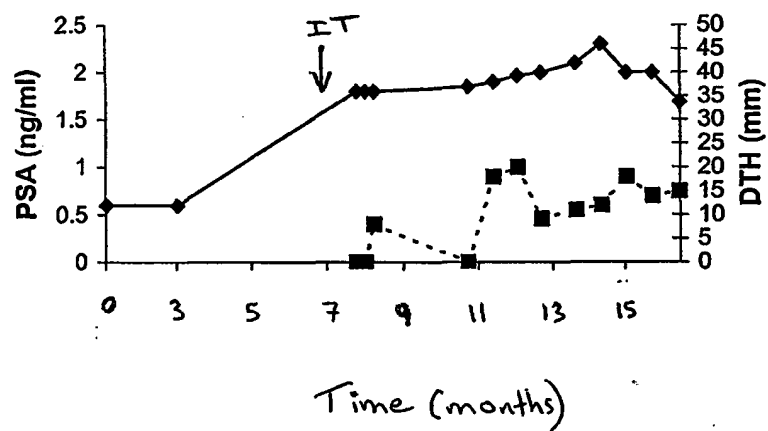


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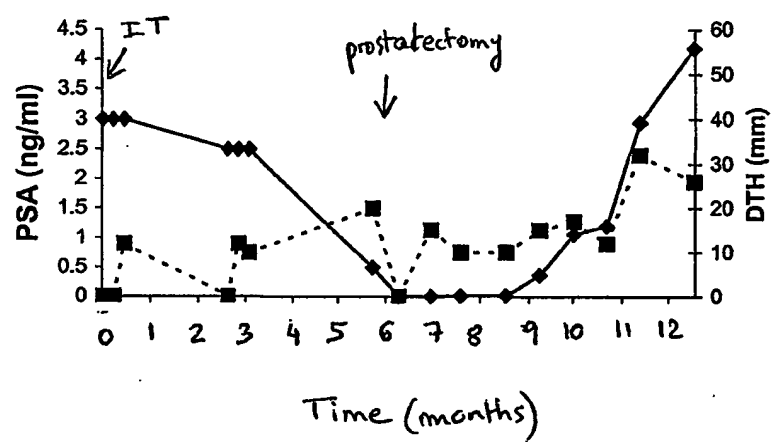


Figure 6

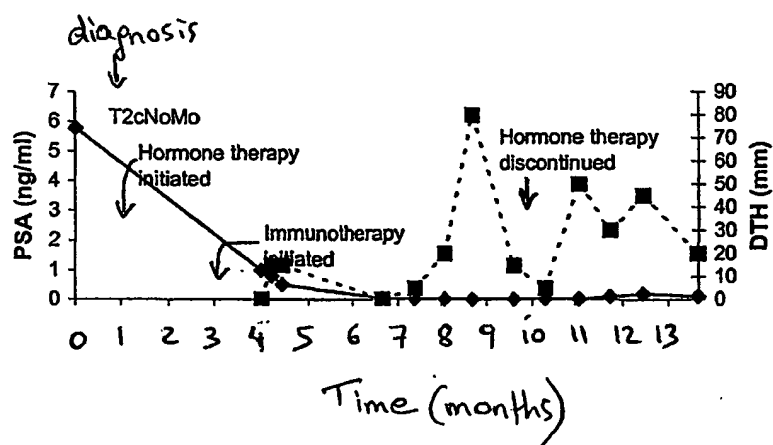


Figure 7

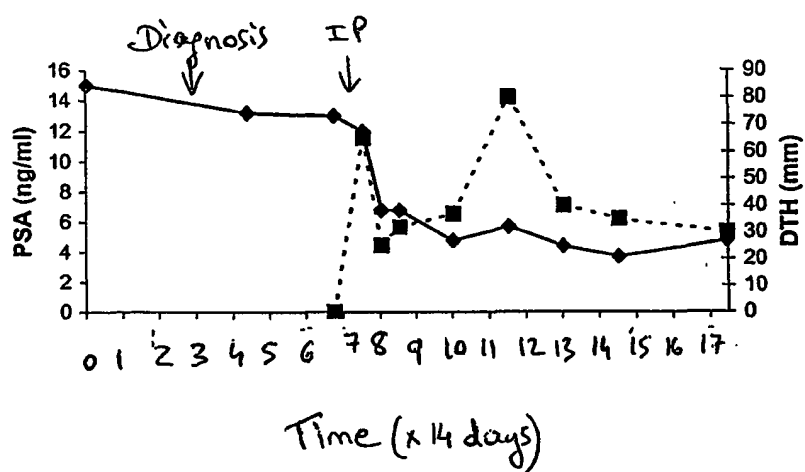


Figure 8

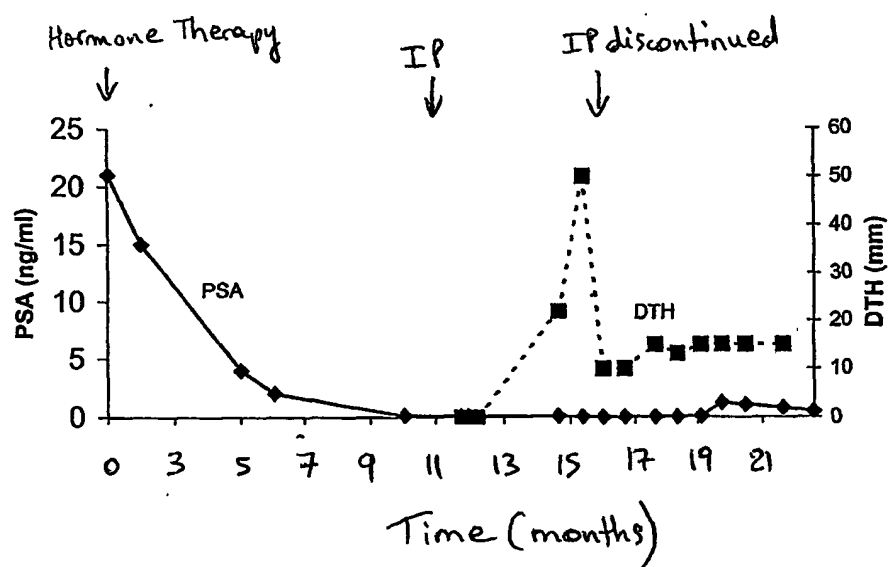


Figure 9

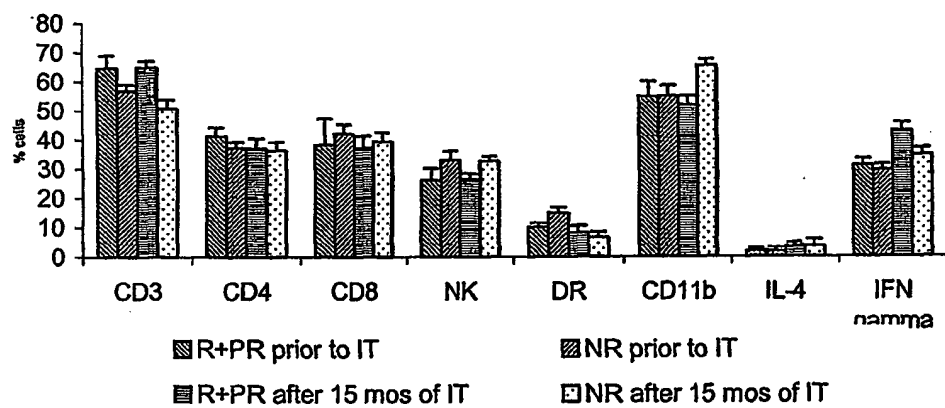


Figure 10

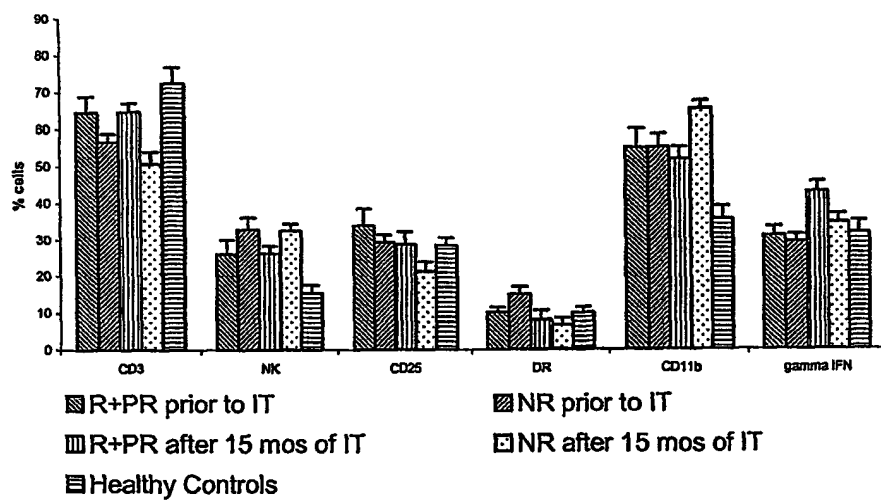
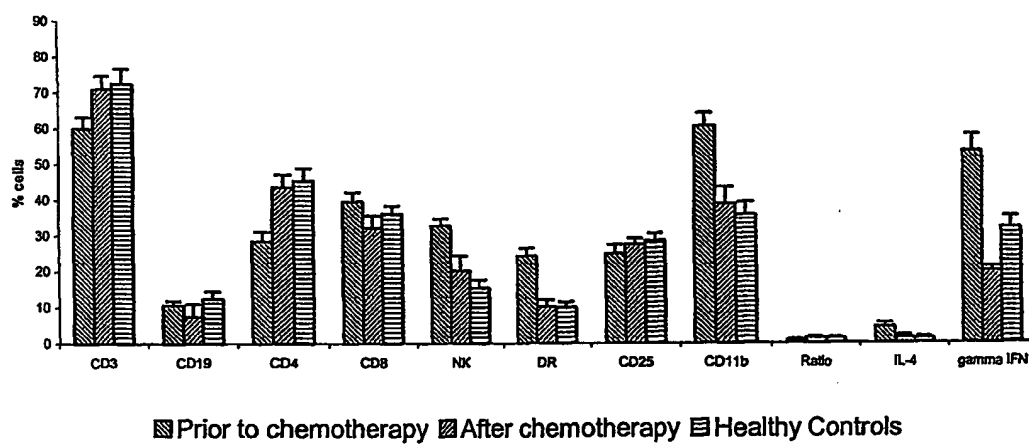




Figure 11



- 1 -

## SEQUENCE LISTING

<110> MINCHEFF, MILCHO  
LOUKINOV, DMITRI  
ZOUBAK, SERGUEI

<120> METHODS AND COMPOSITIONS FOR INDUCING CELL MEDIATED  
IMMUNE RESPONSES

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<140> not yet assigned

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Asp Arg Pro Phe Tyr Arg His Val Ile Tyr Ala Pro Ser Ser His Asn	
685 690 695	
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 Ser Tyr Pro Asp Gly Trp Asn Leu Pro Gly Gly Gly Val Gln Arg Gly  
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Cys Glu Lys His Ser Gln Pro Trp Gln Val Leu Val Ala Ser Arg Gly	
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agg gca gtc tgc ggc ggt gtt ctg gtg cac ccc cag tgg gtc ctc aca	144
Arg Ala Val Cys Gly Gly Val Leu Val His Pro Gln Trp Val Leu Thr	
35 40 45	
gct gcc cac tgc atc agg aac aaa agc gtg atc ttg ctg ggt cgg cac	192
Ala Ala His Cys Ile Arg Asn Lys Ser Val Ile Leu Leu Gly Arg His	
50 55 60	
agc ctg ttt cat cct gaa gac aca ggc cag gta ttt cag gtc agc cac	240
Ser Leu Phe His Pro Glu Asp Thr Gly Gln Val Phe Gln Val Ser His	
65 70 75	
agc ttc cca cac ccg ctc tac gat atg agc ctc ctg aag aat cga ttc	288
Ser Phe Pro His Pro Leu Tyr Asp Met Ser Leu Leu Lys Asn Arg Phe	
80 85 90	
ctc agg cca ggt gat gac tcc agc cac gac ctc atg ctg ctc cgc ctg	336
Leu Arg Pro Gly Asp Asp Ser Ser His Asp Leu Met Leu Leu Arg Leu	
95 100 105 110	
tca gag cct gcc gag ctc acg gat gct gtg aag gtc atg gac ctg ccc	384
Ser Glu Pro Ala Glu Leu Thr Asp Ala Val Lys Val Met Asp Leu Pro	
115 120 125	
acc cag gag cca gca ctg ggg acc acc tgc tac gcc tca ggc tgg ggc	432
Thr Gln Glu Pro Ala Leu Gly Thr Thr Cys Tyr Ala Ser Gly Trp Gly	
130 135 140	
agc att gaa cca gag gag ttc ttg acc cca aag aaa ctt cag tgt gtg	480
Ser Ile Glu Pro Glu Glu Phe Leu Thr Pro Lys Lys Leu Gln Cys Val	
145 150 155	
gac ctc cat gtt att tcc aat gac gtg tgt gcg caa gtt cac cct cag	528
Asp Leu His Val Ile Ser Asn Asp Val Cys Ala Gln Val His Pro Gln	
160 165 170	
aag gtg acc aag ttc atg ctg tgt gct gga cgc tgg aca ggg ggc aaa	576
Lys Val Thr Lys Phe Met Leu Cys Ala Gly Arg Trp Thr Gly Gly Lys	
175 180 185 190	
agc acc tgc tcg ggt gat tct ggg ggc cca ctt gtc tgt aat ggt gtg	624
Ser Thr Cys Ser Gly Asp Ser Gly Gly Pro Leu Val Cys Asn Gly Val	
195 200 205	
ctt caa ggt atc acg tca tgg ggc agt gaa cca tgt gcc ctg ccc gaa	672
Leu Gln Gly Ile Thr Ser Trp Gly Ser Glu Pro Cys Ala Leu Pro Glu	

- 9 -

210	215	220	
agg cct tcc ctg tac acc aag	gtg gtg cat tac cgg aag	tgg atc aag	720
Arg Pro Ser Leu Tyr Thr Lys	Val Val His Tyr Arg Lys	Trp Ile Lys	
225	230	235	
gac acc atc gtg gcc aac ccc tga			744
Asp Thr Ile Val Ala Asn Pro			
240	245		

&lt;210&gt; 4

&lt;211&gt; 245

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens-

&lt;400&gt; 4

Met Ala Pro Leu Ile Leu Ser Arg Ile Val Gly Gly Trp Glu Cys Glu	
1 5 10 15	
Lys His Ser Gln Pro Trp Gln Val Leu Val Ala Ser Arg Gly Arg Ala	
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Val Cys Gly Gly Val Leu Val His Pro Gln Trp Val Leu Thr Ala Ala	
35 40 45	
His Cys Ile Arg Asn Lys Ser Val Ile Leu Leu Gly Arg His Ser Leu	
50 55 60	
Phe His Pro Glu Asp Thr Gly Gln Val Phe Gln Val Ser His Ser Phe	
65 70 75 80	
Pro His Pro Leu Tyr Asp Met Ser Leu Leu Lys Asn Arg Phe Leu Arg	
85 90 95	
Pro Gly Asp Asp Ser Ser His Asp Leu Met Leu Leu Arg Leu Ser Glu	
100 105 110	
Pro Ala Glu Leu Thr Asp Ala Val Lys Val Met Asp Leu Pro Thr Gln	
115 120 125	
Glu Pro Ala Leu Gly Thr Thr Cys Tyr Ala Ser Gly Trp Gly Ser Ile	
130 135 140	
Glu Pro Glu Glu Phe Leu Thr Pro Lys Lys Leu Gln Cys Val Asp Leu	
145 150 155 160	
His Val Ile Ser Asn Asp Val Cys Ala Gln Val His Pro Gln Lys Val	
165 170 175	
Thr Lys Phe Met Leu Cys Ala Gly Arg Trp Thr Gly Gly Lys Ser Thr	
180 185 190	
Cys Ser Gly Asp Ser Gly Gly Pro Leu Val Cys Asn Gly Val Leu Gln	
195 200 205	

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Gly Ile Thr Ser Trp Gly Ser Glu Pro Cys Ala Leu Pro Glu Arg Pro  
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Ser Leu Tyr Thr Lys Val Val His Tyr Arg Lys Trp Ile Lys Asp Thr  
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Ile Val Ala Asn Pro  
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<210> 5

<211> 2019

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<213> Homo sapiens

<220>

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<222> (7)..(2016)

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 1 5 10

gag ggg aag gag gtg ctt cta ctt gtc cac aat ctg ccc cag cat ctt 96  
 Glu Gly Lys Glu Val Leu Leu Leu Val His Asn Leu Pro Gln His Leu  
 15 20 25 30

ttt ggc tac agc tgg tac aaa ggt gaa aga gtg gat ggc aac cgt caa 144  
 Phe Gly Tyr Ser Trp Tyr Lys Gly Glu Arg Val Asp Gly Asn Arg Gln  
 35 40 45

att ata gga tat gta ata gga act caa caa gct acc cca ggg ccc gca 192  
 Ile Ile Gly Tyr Val Ile Gly Thr Gln Gln Ala Thr Pro Gly Pro Ala  
 50 55 60

tac agt ggt cga gag ata ata tac ccc aat gca tcc ctg ctg atc cag 240  
 Tyr Ser Gly Arg Glu Ile Ile Tyr Pro Asn Ala Ser Leu Leu Ile Gln  
 65 70 75

aac atc atc cag aat gac aca gga ttc tac acc cta cac gtc ata aag 288  
 Asn Ile Ile Gln Asn Asp Thr Gly Phe Tyr Thr Leu His Val Ile Lys  
 80 85 90

tca gat ctt gtg aat gaa gaa gca act ggc cag ttc cgg gta tac ccg 336  
 Ser Asp Leu Val Asn Glu Glu Ala Thr Gly Gln Phe Arg Val Tyr Pro  
 95 100 105 110

gag ctg ccc aag ccc tcc atc tcc agc aac aac tcc aaa ccc gtg gag 384  
 Glu Leu Pro Lys Pro Ser Ile Ser Ser Asn Asn Ser Lys Pro Val Glu  
 115 120 125

gac aag gat gct gtg gcc ttc acc tgt gaa cct gag act cag gac gca 432  
 Asp Lys Asp Ala Val Ala Phe Thr Cys Glu Pro Glu Thr Gln Asp Ala  
 130 135 140

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acc tac ctg tgg tgg gta aac aat cag agc ctc ccg gtc agt ccc agg	480
Thr Tyr Leu Trp Trp Val Asn Asn Gln Ser Leu Pro Val Ser Pro Arg	
145 150 155	
ctg cag ctg tcc aat ggc aac agg acc ctc act cta ttc aat gtc aca	528
Leu Gln Leu Ser Asn Gly Asn Arg Thr Leu Thr Leu Phe Asn Val Thr	
160 165 170	
aga aat gac aca gca agc tac aaa tgt gaa acc cag aac cca gtg agt	576
Arg Asn Asp Thr Ala Ser Tyr Lys Cys Glu Thr Gln Asn Pro Val Ser	
175 180 185 190	
gcc agg cgc agt gat tca gtc atc ctg aat gtc ctc tat ggc ccg gat	624
Ala Arg Arg Ser Asp Ser Val Ile Leu Asn Val Leu Tyr Gly Pro Asp	
195 200 205	
gcc ccc acc att tcc cct cta aac aca tct tac aga tca ggg gaa aat	672
Ala Pro Thr Ile Ser Pro Leu Asn Thr Ser Tyr Arg Ser Gly Glu Asn	
210 215 220	
ctg aac ctc tcc tgc cac gca gcc tct aac cca cct gca cag tac tct	720
Leu Asn Leu Ser Cys His Ala Ala Ser Asn Pro Pro Ala Gln Tyr Ser	
225 230 235	
tgg ttt gtc aat ggg act ttc cag caa tcc acc caa gag ctc ttt atc	768
Trp Phe Val Asn Gly Thr Phe Gln Gln Ser Thr Gln Glu Leu Phe Ile	
240 245 250	
ccc aac atc act gtg aat aat agt gga tcc tat acg tgc caa gcc cat	816
Pro Asn Ile Thr Val Asn Asn Ser Gly Ser Tyr Thr Cys Gln Ala His	
255 260 265 270	
aac tca gac act ggc ctc aat agg acc aca gtc acg acg atc aca gtc	864
Asn Ser Asp Thr Gly Leu Asn Arg Thr Thr Val Thr Thr Ile Thr Val	
275 280 285	
tat gca gag cca ccc aaa ccc ttc atc acc agc aac aac tcc aac ccc	912
Tyr Ala Glu Pro Pro Lys Pro Phe Ile Thr Ser Asn Asn Ser Asn Pro	
290 295 300	
gtg gag gat gag gat gct gta gcc tta acc tgt gaa cct gag att cag	960
Val Glu Asp Glu Asp Ala Val Ala Leu Thr Cys Glu Pro Glu Ile Gln	
305 310 315	
aac aca acc tac ctg tgg tgg gta aat aat cag agc ctc ccg gtc agt	1008
Asn Thr Thr Tyr Leu Trp Trp Val Asn Asn Gln Ser Leu Pro Val Ser	
320 325 330	
ccc agg ctg cag ctg tcc aat gac aac agg acc ctc act cta ctc agt	1056
Pro Arg Leu Gln Leu Ser Asn Asp Asn Arg Thr Leu Thr Leu Leu Ser	
335 340 345 350	
gtc aca agg aat gat gta gga ccc tat gag tgt gga atc cag aac gaa	1104
Val Thr Arg Asn Asp Val Gly Pro Tyr Glu Cys Gly Ile Gln Asn Glu	
355 360 365	

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tta agt gtt gac cac agc gac cca gtc atc ctg aat gtc ctc tat ggc Leu Ser Val Asp His Ser Asp Pro Val Ile Leu Asn Val Leu Tyr Gly 370 375 380	1152
cca gac gac ccc acc att tcc ccc tca tac acc tat tac cgt cca ggg Pro Asp Asp Pro Thr Ile Ser Pro Ser Tyr Thr Tyr Tyr Arg Pro Gly 385 390 395	1200
gtg aac ctc agc ctc tcc tgc cat gca gcc tct aac cca cct gca cag Val Asn Leu Ser Leu Ser Cys His Ala Ala Ser Asn Pro Pro Ala Gln 400 405 410	1248
tat tct tgg ctg att gat ggg aac atc cag caa cac aca caa gag ctc Tyr Ser Trp Leu Ile Asp Gly Asn Ile Gln Gln His Thr Gln Glu Leu 415 420 425 430	1296
ttt atc tcc aac atc act gag aag aac agc gga ctc tat acc tgc cag Phe Ile Ser Asn Ile Thr Glu Lys Asn Ser Gly Leu Tyr Thr Cys Gln 435 440 445	1344
gcc aat aac tca gcc agt ggc cac agc agg act aca gtc aag aca atc Ala Asn Asn Ser Ala Ser Gly His Ser Arg Thr Thr Val Lys Thr Ile 450 455 460	1392
aca gtc tct gcg gag ctg ccc aag ccc tcc atc tcc agc aac aac tcc Thr Val Ser Ala Glu Leu Pro Lys Pro Ser Ile Ser Ser Asn Asn Ser 465 470 475	1440
aaa ccc gtg gag gac aag gat gct gtg gcc ttc acc tgt gaa cct gag Lys Pro Val Glu Asp Lys Asp Ala Val Ala Phe Thr Cys Glu Pro Glu 480 485 490	1488
gct cag aac aca acc tac ctg tgg tgg gta aat ggt cag agc ctc cca Ala Gln Asn Thr Thr Tyr Leu Trp Trp Val Asn Gly Gln Ser Leu Pro 495 500 505 510	1536
gtc agt ccc agg ctg cag ctg tcc aat ggc aac agg acc ctc act cta Val Ser Pro Arg Leu Gln Leu Ser Asn Gly Asn Arg Thr Leu Thr Leu 515 520 525	1584
ttc aat gtc aca aga aat gac gca aga gcc tat gta tgt gga atc cag Phe Asn Val Thr Arg Asn Asp Ala Arg Ala Tyr Val Cys Gly Ile Gln 530 535 540	1632
aac tca gtg agt gca aac cgc agt gac cca gtc acc ctg gat gtc ctc Asn Ser Val Ser Ala Asn Arg Ser Asp Pro Val Thr Leu Asp Val Leu 545 550 555	1680
tat ggg ccg gac acc ccc atc att tcc ccc cca gac tcg tct tac ctt Tyr Gly Pro Asp Thr Pro Ile Ile Ser Pro Pro Asp Ser Ser Tyr Leu 560 565 570	1728
tcg gga gcg aac ctc aac ctc tcc tgc cac tcg gcc tct aac cca tcc Ser Gly Ala Asn Leu Asn Leu Ser Cys His Ser Ala Ser Asn Pro Ser 575 580 585 590	1776

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ccg cag tat tct tgg cgt atc aat ggg ata ccg cag caa cac aca caa 1824  
 Pro Gln Tyr Ser Trp Arg Ile Asn Gly Ile Pro Gln Gln His Thr Gln  
                   595                  600                  605

gtt ctc ttt atc gcc aaa atc acg oca aat aat aac ggg acc tat gcc 1872  
 Val Leu Phe Ile Ala Lys Ile Thr Pro Asn Asn Asn Gly Thr Tyr Ala  
                   610                  615                  620

tgt ttt gtc tct aac ttg gct act ggc cgc aat aat tcc ata gtc aag 1920  
 Cys Phe Val Ser Asn Leu Ala Thr Gly Arg Asn Asn Ser Ile Val Lys  
                   625                  630                  635

agc atc aca gtc tct gca tct gga act tct cct ggt ctc tca gct ggg 1968  
 Ser Ile Thr Val Ser Ala Ser Gly Thr Ser Pro Gly Leu Ser Ala Gly  
                   640                  645                  650

gcc act gtc ggc atc atg att gga gtg ctg gtt ggg gtt gct ctg ata 2016  
 Ala Thr Val Gly Ile Met Ile Gly Val Leu Val Gly Val Ala Leu Ile  
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tag 2019

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 <213> Homo sapiens

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                   20                  25                  30

Tyr Ser Trp Tyr Lys Gly Glu Arg Val Asp Gly Asn Arg Gln Ile Ile  
                   35                  40                  45

Gly Tyr Val Ile Gly Thr Gln Gln Ala Thr Pro Gly Pro Ala Tyr Ser  
                   50                  55                  60

Gly Arg Glu Ile Ile Tyr Pro Asn Ala Ser Leu Leu Ile Gln Asn Ile  
                   65                  70                  75                  80

Ile Gln Asn Asp Thr Gly Phe Tyr Thr Leu His Val Ile Lys Ser Asp  
                   85                  90                  95

Leu Val Asn Glu Glu Ala Thr Gly Gln Phe Arg Val Tyr Pro Glu Leu  
                   100                  105                  110

Pro Lys Pro Ser Ile Ser Ser Asn Asn Ser Lys Pro Val Glu Asp Lys  
                   115                  120                  125

Asp Ala Val Ala Phe Thr Cys Glu Pro Glu Thr Gln Asp Ala Thr Tyr  
                   130                  135                  140

- 14 -

Leu Trp Trp Val Asn Asn Gln Ser Leu Pro Val Ser Pro Arg Leu Gln  
 145 150 155 160  
 Leu Ser Asn Gly Asn Arg Thr Leu Thr Leu Phe Asn Val Thr Arg Asn  
 165 170 175  
 Asp Thr Ala Ser Tyr Lys Cys Glu Thr Gln Asn Pro Val Ser Ala Arg  
 180 185 190  
 Arg Ser Asp Ser Val Ile Leu Asn Val Leu Tyr Gly Pro Asp Ala Pro  
 195 200 205  
 Thr Ile Ser Pro Leu Asn Thr Ser Tyr Arg Ser Gly Glu Asn Leu Asn  
 210 215 220  
 Leu Ser Cys His Ala Ala Ser Asn Pro Pro Ala Gln Tyr Ser Trp Phe  
 225 230 235 240  
 Val Asn Gly Thr Phe Gln Gln Ser Thr Gln Glu Leu Phe Ile Pro Asn  
 245 250 255  
 Ile Thr Val Asn Asn Ser Gly Ser Tyr Thr Cys Gln Ala His Asn Ser  
 260 265 270  
 Asp Thr Gly Leu Asn Arg Thr Thr Val Thr Thr Ile Thr Val Tyr Ala  
 275 280 285  
 Glu Pro Pro Lys Pro Phe Ile Thr Ser Asn Asn Ser Asn Pro Val Glu  
 290 295 300  
 Asp Glu Asp Ala Val Ala Leu Thr Cys Glu Pro Glu Ile Gln Asn Thr  
 305 310 315 320  
 Thr Tyr Leu Trp Trp Val Asn Asn Gln Ser Leu Pro Val Ser Pro Arg  
 325 330 335  
 Leu Gln Leu Ser Asn Asp Asn Arg Thr Leu Thr Leu Leu Ser Val Thr  
 340 345 350  
 Arg Asn Asp Val Gly Pro Tyr Glu Cys Gly Ile Gln Asn Glu Leu Ser  
 355 360 365  
 Val Asp His Ser Asp Pro Val Ile Leu Asn Val Leu Tyr Gly Pro Asp  
 370 375 380  
 Asp Pro Thr Ile Ser Pro Ser Tyr Thr Tyr Tyr Arg Pro Gly Val Asn  
 385 390 395 400  
 Leu Ser Leu Ser Cys His Ala Ala Ser Asn Pro Pro Ala Gln Tyr Ser  
 405 410 415  
 Trp Leu Ile Asp Gly Asn Ile Gln Gln His Thr Gln Glu Leu Phe Ile  
 420 425 430  
 Ser Asn Ile Thr Glu Lys Asn Ser Gly Leu Tyr Thr Cys Gln Ala Asp  
 435 440 445

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Asn Ser Ala Ser Gly His Ser Arg Thr Thr Val Lys Thr Ile Thr Val  
 450 455 460  
 Ser Ala Glu Leu Pro Lys Pro Ser Ile Ser Ser Asn Asn Ser Lys Pro  
 465 470 475 480  
 Val Glu Asp Lys Asp Ala Val Ala Phe Thr Cys Glu Pro Glu Ala Gln  
 485 490 495  
 Asn Thr Thr Tyr Leu Trp Trp Val Asn Gly Gln Ser Leu Pro Val Ser  
 500 505 510  
 Pro Arg Leu Gln Leu Ser Asn Gly Asn Arg Thr Leu Thr Leu Phe Asn  
 515 520 525  
 Val Thr Arg Asn Asp Ala Arg Ala Tyr Val Cys Gly Ile Gln Asn Ser  
 530 535 540  
 Val Ser Ala Asn Arg Ser Asp Pro Val Thr Leu Asp Val Leu Tyr Gly  
 545 550 555 560  
 Pro Asp Thr Pro Ile Ile Ser Pro Pro Asp Ser Ser Tyr Leu Ser Gly  
 565 570 575  
 Ala Asn Leu Asn Leu Ser Cys His Ser Ala Ser Asn Pro Ser Pro Gln  
 580 585 590  
 Tyr Ser Trp Arg Ile Asn Gly Ile Pro Gln Gln His Thr Gln Val Leu  
 595 600 605  
 Phe Ile Ala Lys Ile Thr Pro Asn Asn Asn Gly Thr Tyr Ala Cys Phe  
 610 615 620  
 Val Ser Asn Leu Ala Thr Gly Arg Asn Asn Ser Ile Val Lys Ser Ile  
 625 630 635 640  
 Thr Val Ser Ala Ser Gly Thr Ser Pro Gly Leu Ser Ala Gly Ala Thr  
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 Val Gly Ile Met Ile Gly Val Leu Val Gly Val Ala Leu Ile  
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&lt;210&gt; 7

&lt;211&gt; 3678

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (7)..(3675)

&lt;400&gt; 7

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48



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15 20 25 30			
acc tac ctg ccc acc aat gcc agc ctg tcc ttc ctg cag gat atc cag	144		
Thr Tyr Leu Pro Thr Asn Ala Ser Leu Ser Phe Leu Gln Asp Ile Gln			
35 40 45			
gag gtg cag ggc tac gtg ctc atc gct cac aac caa gtg agg cag gtc	192		
Glu Val Gln Gly Tyr Val Leu Ile Ala His Asn Gln Val Arg Gln Val			
50 55 60			
cca ctg cag agg ctg cgg att gtg cga gcc acc cag ctc ttt gag gac	240		
Pro Leu Gln Arg Leu Arg Ile Val Arg Gly Thr Gln Leu Phe Glu Asp			
65 70 75			
aac tat gcc ctg gcc gtg cta gac aat gga gac ccg ctg aac aat acc	288		
Asn Tyr Ala Leu Ala Val Leu Asp Asn Gly Asp Pro Leu Asn Asn Thr			
80 85 90			
acc cct gtc aca ggg gcc tcc cca gga gcc ctg cgg gag ctg cag ctt	336		
Thr Pro Val Thr Gly Ala Ser Pro Gly Gly Leu Arg Glu Leu Gln Leu			
95 100 105 110			
cga agc ctc aca gag atc ttg aaa gga ggg gtc ttg atc cag cgg aac	384		
Arg Ser Leu Thr Glu Ile Leu Lys Gly Gly Val Leu Ile Gln Arg Asn			
115 120 125			
ccc cag ctc tgc tac cag gac acg att ttg tgg aag gac atc ttc cac	432		
Pro Gln Leu Cys Tyr Gln Asp Thr Ile Leu Trp Lys Asp Ile Phe His			
130 135 140			
aag aac aac cag ctg gct ctc aca ctg ata gac acc aac cgc tct cgg	480		
Lys Asn Asn Gln Leu Ala Leu Thr Leu Ile Asp Thr Asn Arg Ser Arg			
145 150 155			
gcc tgc cac ccc tgt tct ccg atg tgt aag ggc tcc cgc tgc tgg gga	528		
Ala Cys His Pro Cys Ser Pro Met Cys Lys Gly Ser Arg Cys Trp Gly			
160 165 170			
gag agt tct gag gat tgt cag agc ctg acg cgc act gtc tgt gcc ggt	576		
Glu Ser Ser Glu Asp Cys Gln Ser Leu Thr Arg Thr Val Cys Ala Gly			
175 180 185 190			
ggc tgt gcc cgc tgc aag ggg cca ctg ccc act gac tgc tgc cat gag	624		
Gly Cys Ala Arg Cys Lys Gly Pro Leu Pro Thr Asp Cys Cys His Glu			
195 200 205			
cag tgt gct gcc ggc tgc acg ggc ccc aag cac tct gac tgc ctg gcc	672		
Gln Cys Ala Ala Gly Cys Thr Gly Pro Lys His Ser Asp Cys Leu Ala			
210 215 220			
tgc ctc cac ttc aac cac agt ggc atc tgt gag ctg cac tgc cca gcc	720		
Cys Leu His Phe Asn His Ser Gly Ile Cys Glu Leu His Cys Pro Ala			

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225	230	235	
ctg gtc acc tac aac aca gac acg ttt gag tcc atg ccc aat ccc gag			768
Leu Val Thr Tyr Asn Thr Asp Thr Phe Glu Ser Met Pro Asn Pro Glu			
240	245	250	
ggc cgg tat aca ttc ggc gcc agc tgt gtg act gcc tgt ccc tac aac			816
Gly Arg Tyr Thr Phe Gly Ala Ser Cys Val Thr Ala Cys Pro Tyr Asn			
255	260	265	270
tac ctt tct acg gac gtg gga tcc tgc acc ctc gtc tgc ccc ctg cac			864
Tyr Leu Ser Thr Asp Val Gly Ser Cys Thr Leu Val Cys Pro Leu His			
275	280		285
aac caa gag gtg aca gca gag gat gga aca cag cgg tgt gag aag tgc			912
Asn Gln Glu Val Thr Ala Glu Asp Gly Thr Gln Arg Cys Glu Lys Cys			
290	295		300
agc aag ccc tgt gcc cga gtg tgc tat ggt ctg ggc atg gag cac ttg			960
Ser Lys Pro Cys Ala Arg Val Cys Tyr Gly Leu Gly Met Glu His Leu			
305	310		315
cga gag gtg agg gca gtt acc agt gcc aat atc cag gag ttt gct ggc			1008
Arg Glu Val Arg Ala Val Thr Ser Ala Asn Ile Gln Glu Phe Ala Gly			
320	325		330
tgc aag aag atc ttt ggg agc ctg gca ttt ctg ccg gag agc ttt gat			1056
Cys Lys Lys Ile Phe Gly Ser Leu Ala Phe Leu Pro Glu Ser Phe Asp			
335	340		345
ggg gac cca gcc tcc aac act gcc ccg ctc cag cca gag cag ctc caa			1104
Gly Asp Pro Ala Ser Asn Thr Ala Pro Leu Gln Pro Glu Gln Leu Gln			
355	360		365
gtg ttt gag act ctg gaa gag atc aca ggt tac cta tac atc tca gca			1152
Val Phe Glu Thr Leu Glu Glu Ile Thr Gly Tyr Leu Tyr Ile Ser Ala			
370	375		380
tgg ccg gac agc ctg cct gac ctc agc gtc ttc cag aac ctg caa gta			1200
Trp Pro Asp Ser Leu Pro Asp Leu Ser Val Phe Gln Asn Leu Gln Val			
385	390		395
atc cgg gga cga att ctg cac aat ggc gcc tac tcg ctg acc ctg caa			1248
Ile Arg Gly Arg Ile Leu His Asn Gly Ala Tyr Ser Leu Thr Leu Gln			
400	405		410
ggg ctg ggc atc agc tgg ctg ggg ctg cgc tca ctg agg gaa ctg ggc			1296
Gly Leu Gly Ile Ser Trp Leu Gly Leu Arg Ser Leu Arg Glu Leu Gly			
415	420		425
agt gga ctg gcc ctc atc cac cat aac acc cac ctc tgc ttc gtg cac			1344
Ser Gly Leu Ala Leu Ile His His Asn Thr His Leu Cys Phe Val His			
435	440		445
acg gtg ccc tgg gac cag ctc ttt cgg aac ccg cac caa gct ctg ctc			1392
Thr Val Pro Trp Asp Gln Leu Phe Arg Asn Pro His Gln Ala Leu Leu			

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450	455	460	
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tgc cac cag ctg tgc gcc cga ggg cac tgc tgg ggt cca ggg ccc acc Cys His Gln Leu Cys Ala Arg Gly His Cys Trp Gly Pro Gly Pro Thr 480 485 490			1488
cag tgt gtc aac tgc agc cag ttc ctt cgg ggc cag gag tgc gtg gag Gln Cys Val Asn Cys Ser Gln Phe Leu Arg Gly Gln Glu Cys Val Glu 495 500 505 510			1536
gaa tgc cga gta ctg cag ggg ctc ccc agg gag tat gtg aat gcc agg Glu Cys Arg Val Leu Gln Gly Leu Pro Arg Glu Tyr Val Asn Ala Arg 515 520 525			1584
cac tgt ttg ccg tgc cac cct gag tgt cag ccc cag aat ggc tca gtg His Cys Leu Pro Cys His Pro Glu Cys Gln Pro Gln Asn Gly Ser Val 530 535 540			1632
acc tgt ttt gga ccg gag gct gac cag tgt gtg gcc tgt gcc cac tat Thr Cys Phe Gly Pro Glu Ala Asp Gln Cys Val Ala Cys Ala His Tyr 545 550 555			1680
aag gac cct ccc ttc tgc gtg gcc cgc tgc ccc agc ggt gtg aaa cct Lys Asp Pro Pro Phe Cys Val Ala Arg Cys Pro Ser Gly Val Lys Pro 560 565 570			1728
gac ctc tcc tac atg ccc atc tgg aag ttt cca gat gag gag ggc gca Asp Leu Ser Tyr Met Pro Ile Trp Lys Phe Pro Asp Glu Glu Gly Ala 575 580 585 590			1776
tgc cag cct tgc ccc atc aac tgc acc cac tcc tgt gtg gac ctg gat Cys Gln Pro Cys Pro Ile Asn Cys Thr His Ser Cys Val Asp Leu Asp 595 600 605			1824
gac aag ggc tgc ccc gcc gag cag aga gcc agc cct ctg acg tcc atc Asp Lys Gly Cys Pro Ala Glu Gln Arg Ala Ser Pro Leu Thr Ser Ile 610 615 620			1872
gtc tct gcg gtg gtt ggc att ctg ctg gtc gtg gtc ttg ggg gtg gtc Val Ser Ala Val Val Gly Ile Leu Leu Val Val Val Leu Gly Val Val 625 630 635			1920
ttt ggg atc ctc atc aag cga cgg cag cag aag atc cgg aag tac acg Phe Gly Ile Leu Ile Lys Arg Arg Gln Gln Lys Ile Arg Lys Tyr Thr 640 645 650			1968
atg cgg aga ctg ctg cag gaa acg gag ctg gtg gag ccg ctg aca cct Met Arg Arg Leu Leu Gln Glu Thr Glu Leu Val Glu Pro Leu Thr Pro 655 660 665 670			2016
agc gga gcg atg ccc aac cag gcg cag atg cgg atc ctg aaa gag acg Ser Gly Ala Met Pro Asn Gln Ala Gln Met Arg Ile Leu Lys Glu Thr 2064			

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675	680	685	
gag ctg agg aag gtg aag gtg ctt gga tct ggc gct ttt ggc aca gtc Glu Leu Arg Lys Val Lys Val Leu Gly Ser Gly Ala Phe Gly Thr Val 690 695 700			2112
tac aag ggc atc tgg atc cct gat ggg gag aat gtg aaa att cca gtg Tyr Lys Gly Ile Trp Ile Pro Asp Gly Glu Asn Val Lys Ile Pro Val 705 710 715			2160
gcc atc aaa gtg ttg agg gaa aac aca tcc ccc aaa gcc aac aaa gaa Ala Ile Lys Val Leu Arg Glu Asn Thr Ser Pro Lys Ala Asn Lys Glu 720 725 730			2208
atc tta gac gaa gca tac gtg atg gct ggt gtg ggc tcc cca tat gtc Ile Leu Asp Glu Ala Tyr Val Met Ala Gly Val Gly Ser Pro Tyr Val 735 740 745 750			2256
tcc cgc ctt ctg ggc atc tgc ctg aca tcc acg gtg cag ctg gtg aca Ser Arg Leu Leu Gly Ile Cys Leu Thr Ser Thr Val Gln Leu Val Thr 755 760 765			2304
cag ctt atg ccc tat ggc tgc ctc tta gac cat gtc cgg gaa aac cgc Gln Leu Met Pro Tyr Gly Cys Leu Leu Asp His Val Arg Glu Asn Arg 770 775 780			2352
gga cgc ctg ggc tcc cag gac ctg ctg aac tgg tgt atg cag att gcc Gly Arg Leu Gly Ser Gln Asp Leu Leu Asn Trp Cys Met Gln Ile Ala 785 790 795			2400
aag ggg atg agc tac ctg gag gat gtg cgg ctc gta cac agg gac ttg Lys Gly Met Ser Tyr Leu Glu Asp Val Arg Leu Val His Arg Asp Leu 800 805 810			2448
gcc gct cgg aac gtg ctg gtc aag agt ccc aac cat gtc aaa att aca Ala Ala Arg Asn Val Leu Val Lys Ser Pro Asn His Val Lys Ile Thr 815 820 825 830			2496
gac ttc ggg ctg gct cgg ctg ctg gac att gac gag aca gag tac cat Asp Phe Gly Leu Ala Arg Leu Leu Asp Ile Asp Glu Thr Glu Tyr His 835 840 845			2544
gca gat ggg ggc aag gtg ccc atc aag tgg atg gcg ctg gag tcc att Ala Asp Gly Gly Lys Val Pro Ile Lys Trp Met Ala Leu Glu Ser Ile 850 855 860			2592
ctc cgc cgg cgg ttc acc cac cag agt gat gtg tgg agt tat ggt gtg Leu Arg Arg Arg Phe Thr His Gln Ser Asp Val Trp Ser Tyr Gly Val 865 870 875			2640
act gtg tgg gag ctg atg act ttt ggg gcc aaa cct tac gat ggg atc Thr Val Trp Glu Leu Met Thr Phe Gly Ala Lys Pro Tyr Asp Gly Ile 880 885 890			2688
cca gcc cgg gag atc cct gac ctg ctg gaa aag ggg gag cgg ctg ccc Pro Ala Arg Glu Ile Pro Asp Leu Leu Glu Lys Gly Glu Arg Leu Pro			2736

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895	900	905	910	
cag ccc ccc atc tgc acc att gat gtc tac atg atc atg gtc aaa tgt				2784
Gln Pro Pro Ile Cys Thr Ile Asp Val Tyr Met Ile Met Val Lys Cys	915	920	925	
tgg atg att gac tct gaa tgt cgg cca aga ttc cgg gag ttg gtg tct				2832
Trp Met Ile Asp Ser Glu Cys Arg Pro Arg Phe Arg Glu Leu Val Ser	930	935	940	
gaa ttc tcc cgc atg gcc agg gac ccc cag cgc ttt gtg gtc atc cag				2880
Glu Phe Ser Arg Met Ala Arg Asp Pro Gln Arg Phe Val Val Ile Gln	945	950	955	
aat gag gac ttg ggc cca gcc agt ccc ttg gac agc acc ttc tac cgc				2928
Asn Glu Asp Leu Gly Pro Ala Ser Pro Leu Asp Ser Thr Phe Tyr Arg	960	965	970	
tca ctg ctg gag gac gat gac atg ggg gac ctg gtg gat gct gag gag				2976
Ser Leu Leu Glu Asp Asp Asp Met Gly Asp Leu Val Asp Ala Glu Glu	975	980	985	990
tat ctg gta ccc cag cag ggc ttc ttc tgt cca gac cct gcc ccg ggc				3024
Tyr Leu Val Pro Gln Gln Gly Phe Phe Cys Pro Asp Pro Ala Pro Gly	995	1000	1005	
gct ggg ggc atg gtc cac cac agg cac cgc agc tca tct acc agg agt				3072
Ala Gly Gly Met Val His His Arg His Arg Ser Ser Ser Thr Arg Ser	1010	1015	1020	
ggc ggt ggg gac ctg aca cta ggg ctg gag ccc tct gaa gag gag gcc				3120
Gly Gly Gly Asp Leu Thr Leu Gly Leu Glu Pro Ser Glu Glu Glu Ala	1025	1030	1035	
ccc agg tct cca ctg gca ccc tcc gaa ggg gct ggc tcc gat gta ttt				3168
Pro Arg Ser Pro Leu Ala Pro Ser Glu Gly Ala Gly Ser Asp Val Phe	1040	1045	1050	
gat ggt gac ctg gga atg ggg gca gcc aag ggg ctg caa agc ctc ccc				3216
Asp Gly Asp Leu Gly Met Gly Ala Ala Lys Gly Leu Gln Ser Leu Pro	1055	1060	1065	1070
aca cat gac ccc agc cct cta cag cgg tac agt gag gac ccc aca gta				3264
Thr His Asp Pro Ser Pro Leu Gln Arg Tyr Ser Glu Asp Pro Thr Val	1075	1080	1085	
ccc ctg ccc tct gag act gat ggc tac gtt gcc ccc ctg acc tgc agc				3312
Pro Leu Pro Ser Glu Thr Asp Gly Tyr Val Ala Pro Leu Thr Cys Ser	1090	1095	1100	
ccc cag cct gaa tat gtg aac cag cca gat gtt cgg ccc cag ccc cct				3360
Pro Gln Pro Glu Tyr Val Asn Gln Pro Asp Val Arg Pro Gln Pro Pro	1105	1110	1115	
tcg ccc cga gag ggc cct ctg cct gct gcc cga cct gct ggt gcc act				3408
Ser Pro Arg Glu Gly Pro Leu Pro Ala Ala Arg Pro Ala Gly Ala Thr				

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1120	1125	1130	
ctg gaa agg gcc aag act ctc tcc cca ggg aag aat ggg gtc gtc aaa			3456
Leu Glu Arg Ala Lys Thr Leu Ser Pro Gly Lys Asn Gly Val Val Lys			
1135	1140	1145	1150
gac gtt ttt gcc ttt ggg ggt gcc gtg gag aac ccc gag tac ttg aca			3504
Asp Val Phe Ala Phe Gly Gly Ala Val Glu Asn Pro Glu Tyr Leu Thr			
1155	1160		1165
ccc cag gga gga gct gcc cct cag ccc cac cct cct cct gcc ttc agc			3552
Pro Gln Gly Gly Ala Ala Pro Gln Pro His Pro Pro Pro Ala Phe Ser			
1170	1175		1180
cca gcc ttc gac aac ctc tat tac tgg gac cag gac cca cca gag cgg			3600
Pro Ala Phe Asp Asn Leu Tyr Tyr Trp Asp Gln Asp Pro Pro Glu Arg			
1185	1190		1195
ggg gct cca ccc agc acc ttc aaa ggg aca cct acg gca gag aac cca			3648
Gly Ala Pro Pro Ser Thr Phe Lys Gly Thr Pro Thr Ala Glu Asn Pro			
1200	1205		1210
gag tac ctg ggt ctg gac gtg cca gtg tga			3678
Glu Tyr Leu Gly Leu Asp Val Pro Val			
1215	1220		

&lt;210&gt; 8

&lt;211&gt; 1223

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 8

Met Arg Leu Pro Ala Ser Pro Glu Thr His Leu Asp Met Leu Arg His

1

5

10

15

Leu Tyr Gln Gly Cys Gln Val Val Gln Gly Asn Leu Glu Leu Thr Tyr

20

25

30

Leu Pro Thr Asn Ala Ser Leu Ser Phe Leu Gln Asp Ile Gln Glu Val

35

40

45

Gln Gly Tyr Val Leu Ile Ala His Asn Gln Val Arg Gln Val Pro Leu

50

55

60

Gln Arg Leu Arg Ile Val Arg Gly Thr Gln Leu Phe Glu Asp Asn Tyr

65

70

75

80

Ala Leu Ala Val Leu Asp Asn Gly Asp Pro Leu Asn Asn Thr Thr Pro

85

90

95

Val Thr Gly Ala Ser Pro Gly Gly Leu Arg Glu Leu Gln Leu Arg Ser

100

105

110

Leu Thr Glu Ile Leu Lys Gly Gly Val Leu Ile Gln Arg Asn Pro Gln

115

120

125

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Leu Cys Tyr Gln Asp Thr Ile Leu Trp Lys Asp Ile Phe His Lys Asn  
 130 135 140  
 Asn Gln Leu Ala Leu Thr Leu Ile Asp Thr Asn Arg Ser Arg Ala Cys  
 145 150 155 160  
 His Pro Cys Ser Pro Met Cys Lys Gly Ser Arg Cys Trp Gly Glu Ser  
 165 170 175  
 Ser Glu Asp Cys Gln Ser Leu Thr Arg Thr Val Cys Ala Gly Gly Cys  
 180 185 190  
 Ala Arg Cys Lys Gly Pro Leu Pro Thr Asp Cys Cys His Glu Gln Cys  
 195 200 205  
 Ala Ala Gly Cys Thr Gly Pro Lys His Ser Asp Cys Leu Ala Cys Leu  
 210 215 220  
 His Phe Asn His Ser Gly Ile Cys Glu Leu His Cys Pro Ala Leu Val  
 225 230 235 240  
 Thr Tyr Asn Thr Asp Thr Phe Glu Ser Met Pro Asn Pro Glu Gly Arg  
 245 250 255  
 Tyr Thr Phe Gly Ala Ser Cys Val Thr Ala Cys Pro Tyr Asn Tyr Leu  
 260 265 270  
 Ser Thr Asp Val Gly Ser Cys Thr Leu Val Cys Pro Leu His Asn Gln  
 275 280 285  
 Glu Val Thr Ala Glu Asp Gly Thr Gln Arg Cys Glu Lys Cys Ser Lys  
 290 295 300  
 Pro Cys Ala Arg Val Cys Tyr Gly Leu Gly Met Glu His Leu Arg Glu  
 305 310 315 320  
 Val Arg Ala Val Thr Ser Ala Asn Ile Gln Glu Phe Ala Gly Cys Lys  
 325 330 335  
 Lys Ile Phe Gly Ser Leu Ala Phe Leu Pro Glu Ser Phe Asp Gly Asp  
 340 345 350  
 Pro Ala Ser Asn Thr Ala Pro Leu Gln Pro Glu Gln Leu Gln Val Phe  
 355 360 365  
 Glu Thr Leu Glu Glu Ile Thr Gly Tyr Leu Tyr Ile Ser Ala Trp Pro  
 370 375 380  
 Asp Ser Leu Pro Asp Leu Ser Val Phe Gln Asn Leu Gln Val Ile Arg  
 385 390 395 400  
 Gly Arg Ile Leu His Asn Gly Ala Tyr Ser Leu Thr Leu Gln Gly Leu  
 405 410 415  
 Gly Ile Ser Trp Leu Gly Leu Arg Ser Leu Arg Glu Leu Gly Ser Gly

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420										425										430									
Leu	Ala	Leu	Ile	His	His	Asn	Thr	His	Leu	Cys	Phe	Val	His	Thr	Val														
435										440										445									
Pro	Trp	Asp	Gln	Leu	Phe	Arg	Asn	Pro	His	Gln	Ala	Leu	Leu	His	Thr														
450										455										460									
Ala	Asn	Arg	Pro	Glu	Asp	Glu	Cys	Val	Gly	Glu	Gly	Leu	Ala	Cys	His														
465										470										475									
Gln	Leu	Cys	Ala	Arg	Gly	His	Cys	Trp	Gly	Pro	Gly	Pro	Thr	Gln	Cys														
485										490										495									
Val	Asn	Cys	Ser	Gln	Phe	Leu	Arg	Gly	Gln	Glu	Cys	Val	Glu	Glu	Cys														
500										505										510									
Arg	Val	Leu	Gln	Gly	Leu	Pro	Arg	Glu	Tyr	Val	Asn	Ala	Arg	His	Cys														
515										520										525									
Leu	Pro	Cys	His	Pro	Glu	Cys	Gln	Pro	Gln	Asn	Gly	Ser	Val	Thr	Cys														
530										535										540									
Phe	Gly	Pro	Glu	Ala	Asp	Gln	Cys	Val	Ala	Cys	Ala	His	Tyr	Lys	Asp														
545										550										555									
Pro	Pro	Phe	Cys	Val	Ala	Arg	Cys	Pro	Ser	Gly	Val	Lys	Pro	Asp	Leu														
565										570										575									
Ser	Tyr	Met	Pro	Ile	Trp	Lys	Phe	Pro	Asp	Glu	Glu	Gly	Ala	Cys	Gln														
580										585										590									
Pro	Cys	Pro	Ile	Asn	Cys	Thr	His	Ser	Cys	Val	Asp	Leu	Asp	Asp	Lys														
595										600										605									
Gly	Cys	Pro	Ala	Glu	Gln	Arg	Ala	Ser	Pro	Leu	Thr	Ser	Ile	Val	Ser														
610										615										620									
Ala	Val	Val	Gly	Ile	Leu	Leu	Val	Val	Val	Leu	Gly	Val	Val	Phe	Gly														
625										630										635									
Ile	Leu	Ile	Lys	Arg	Arg	Gln	Gln	Lys	Ile	Arg	Lys	Tyr	Thr	Met	Arg														
645										650										655									
Arg	Leu	Leu	Gln	Glu	Thr	Glu	Leu	Val	Glu	Pro	Leu	Thr	Pro	Ser	Gly														
660										665										670									
Ala	Met	Pro	Asn	Gln	Ala	Gln	Met	Arg	Ile	Leu	Lys	Glu	Thr	Glu	Leu														
675										680										685									
Arg	Lys	Val	Lys	Val	Leu	Gly	Ser	Gly	Ala	Phe	Gly	Thr	Val	Tyr	Lys														
690										695										700									
Gly	Ile	Trp	Ile	Pro	Asp	Gly	Glu	Asn	Val	Lys	Ile	Pro	Val	Ala	Ile														
705										710										715									
																				720									



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Lys Val Leu Arg Glu Asn Thr Ser Pro Lys Ala Asn Lys Glu Ile Leu  
 725 730 735  
 Asp Glu Ala Tyr Val Met Ala Gly Val Gly Ser Pro Tyr Val Ser Arg  
 740 745 750  
 Leu Leu Gly Ile Cys Leu Thr Ser Thr Val Gln Leu Val Thr Gln Leu  
 755 760 765  
 Met Pro Tyr Gly Cys Leu Leu Asp His Val Arg Glu Asn Arg Gly Arg  
 770 775 780  
 Leu Gly Ser Gln Asp Leu Leu Asn Trp Cys Met Gln Ile Ala Lys Gly  
 785 790 795 800  
 Met Ser Tyr Leu Glu Asp Val Arg Leu Val His Arg Asp Leu Ala Ala  
 805 810 815  
 Arg Asn Val Leu Val Lys Ser Pro Asn His Val Lys Ile Thr Asp Phe  
 820 825 830  
 Gly Leu Ala Arg Leu Leu Asp Ile Asp Glu Thr Glu Tyr His Ala Asp  
 835 840 845  
 Gly Gly Lys Val Pro Ile Lys Trp Met Ala Leu Glu Ser Ile Leu Arg  
 850 855 860  
 Arg Arg Phe Thr His Gln Ser Asp Val Trp Ser Tyr Gly Val Thr Val  
 865 870 875 880  
 Trp Glu Leu Met Thr Phe Gly Ala Lys Pro Tyr Asp Gly Ile Pro Ala  
 885 890 895  
 Arg Glu Ile Pro Asp Leu Leu Glu Lys Gly Glu Arg Leu Pro Gln Pro  
 900 905 910  
 Pro Ile Cys Thr Ile Asp Val Tyr Met Ile Met Val Lys Cys Trp Met  
 915 920 925  
 Ile Asp Ser Glu Cys Arg Pro Arg Phe Arg Glu Leu Val Ser Glu Phe  
 930 935 940  
 Ser Arg Met Ala Arg Asp Pro Gln Arg Phe Val Val Ile Gln Asn Glu  
 945 950 955 960  
 Asp Leu Gly Pro Ala Ser Pro Leu Asp Ser Thr Phe Tyr Arg Ser Leu  
 965 970 975  
 Leu Glu Asp Asp Asp Met Gly Asp Leu Val Asp Ala Glu Glu Tyr Leu  
 980 985 990  
 Val Pro Gln Gln Gly Phe Phe Cys Pro Asp Pro Ala Pro Gly Ala Gly  
 995 1000 1005  
 Gly Met Val His His Arg His Arg Ser Ser Ser Thr Arg Ser Gly Gly  
 1010 1015 1020

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Gly Asp Leu Thr Leu Gly Leu Glu Pro Ser Glu Glu Glu Ala Pro Arg  
 1025 1030 1035 1040

Ser Pro Leu Ala Pro Ser Glu Gly Ala Gly Ser Asp Val Phe Asp Gly  
 1045 1050 1055

Asp Leu Gly Met Gly Ala Ala Lys Gly Leu Gln Ser Leu Pro Thr His  
 1060 1065 1070

Asp Pro Ser Pro Leu Gln Arg Tyr Ser Glu Asp Pro Thr Val Pro Leu  
 1075 1080 1085

Pro Ser Glu Thr Asp Gly Tyr Val Ala Pro Leu Thr Cys Ser Pro Gln  
 1090 1095 1100

Pro Glu Tyr Val Asn Gln Pro Asp Val Arg Pro Gln Pro Pro Ser Pro  
 1105 1110 1115 1120

Arg Glu Gly Pro Leu Pro Ala Ala Arg Pro Ala Gly Ala Thr Leu Glu  
 1125 1130 1135

Arg Ala Lys Thr Leu Ser Pro Gly Lys Asn Gly Val Val Lys Asp Val  
 1140 1145 1150

Phe Ala Phe Gly Gly Ala Val Glu Asn Pro Glu Tyr Leu Thr Pro Gln  
 1155 1160 1165

Gly Gly Ala Ala Pro Gln Pro His Pro Pro Pro Ala Phe Ser Pro Ala  
 1170 1175 1180

Phe Asp Asn Leu Tyr Tyr Trp Asp Gln Asp Pro Pro Glu Arg Gly Ala  
 1185 1190 1195 1200

Pro Pro Ser Thr Phe Lys Gly Thr Pro Thr Ala Glu Asn Pro Glu Tyr  
 1205 1210 1215

Leu Gly Leu Asp Val Pro Val  
 1220

&lt;210&gt; 9

&lt;211&gt; 855

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (7) .. (852)

&lt;400&gt; 9

gccacc atg gag gag cag gag act gcc tcc tcc tcc tct act cta gtg 48  
 Met Glu Glu Gln Glu Thr Ala Ser Ser Ser Ser Thr Leu Val  
 1 5 10

gaa gtc acc ctg cgg gag gtg cct gct gcc gag tca cca agt cct ccc 96

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Gly Asp Leu Thr Leu Gly Leu Glu Pro Ser Glu Glu Glu Ala Pro Arg  
 1025 1030 1035 1040

Ser Pro Leu Ala Pro Ser Glu Gly Ala Gly Ser Asp Val Phe Asp Gly  
 1045 1050 1055

Asp Leu Gly Met Gly Ala Ala Lys Gly Leu Gln Ser Leu Pro Thr His  
 1060 1065 1070

Asp Pro Ser Pro Leu Gln Arg Tyr Ser Glu Asp Pro Thr Val Pro Leu  
 1075 1080 1085

Pro Ser Glu Thr Asp Gly Tyr Val Ala Pro Leu Thr Cys Ser Pro Gln  
 1090 1095 1100

Pro Glu Tyr Val Asn Gln Pro Asp Val Arg Pro Gln Pro Pro Ser Pro  
 1105 1110 1115 1120

Arg Glu Gly Pro Leu Pro Ala Ala Arg Pro Ala Gly Ala Thr Leu Glu  
 1125 1130 1135

Arg Ala Lys Thr Leu Ser Pro Gly Lys Asn Gly Val Val Lys Asp Val  
 1140 1145 1150

Phe Ala Phe Gly Gly Ala Val Glu Asn Pro Glu Tyr Leu Thr Pro Gln  
 1155 1160 1165

Gly Gly Ala Ala Pro Gln Pro His Pro Pro Pro Ala Phe Ser Pro Ala  
 1170 1175 1180

Phe Asp Asn Leu Tyr Tyr Trp Asp Gln Asp Pro Pro Glu Arg Gly Ala  
 1185 1190 1195 1200

Pro Pro Ser Thr Phe Lys Gly Thr Pro Thr Ala Glu Asn Pro Glu Tyr  
 1205 1210 1215

Leu Gly Leu Asp Val Pro Val  
 1220

<210> 9

<211> 855

<212> DNA

<213> Homo sapiens

<220>

<221> CDS

<222> (7) .. (852)

<400> 9

gccacc atg gag gag cag gag act gcc tcc tcc tcc tct act cta gtg 48  
 Met Glu Glu Gln Glu Thr Ala Ser Ser Ser Ser Thr Leu Val  
 1 5 10

gaa gtc acc ctg cgg gag gtg cct gct gcc gag tca cca agt cct ccc 96

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Glu	Val	Thr	Leu	Arg	Glu	Val	Pro	Ala	Ala	Glu	Ser	Pro	Ser	Pro	Pro		
15					20					25					30		
cac	agt	cct	cag	gga	gcc	tcc	acc	ctc	ccc	act	acc	atc	aac	tat	act	144	
His	Ser	Pro	Gln	Gly	Ala	Ser	Thr	Leu	Pro	Thr	Thr	Ile	Asn	Tyr	Thr		
				35					40					45			
ctc	tgg	agt	caa	tcc	gat	gag	ggc	tcc	agc	aac	gaa	gaa	cag	gaa	ggg	192	
Leu	Trp	Ser	Gln	Ser	Asp	Glu	Gly	Ser	Ser	Asn	Glu	Glu	Gln	Glu	Gly		
			50					55					60				
cca	agc	acc	ttt	cct	gac	ctg	gag	acg	agc	ttc	caa	gta	gca	ctc	agt	240	
Pro	Ser	Thr	Phe	Pro	Asp	Leu	Glu	Thr	Ser	Phe	Gln	Val	Ala	Leu	Ser		
			65				70					75					
agg	aag	atg	gct	gag	ttg	gtt	cat	ttt	ctg	ctc	ctc	aag	tat	cga	gcc	288	
Arg	Lys	Met	Ala	Glu	Leu	Val	His	Phe	Leu	Leu	Leu	Lys	Tyr	Arg	Ala		
	80					85					90						
agg	gag	cca	ttc	aca	aag	gca	gaa	atg	ctg	ggg	agt	gtc	atc	aga	aat	336	
Arg	Glu	Pro	Phe	Thr	Lys	Ala	Glu	Met	Leu	Gly	Ser	Val	Ile	Arg	Asn		
	95				100					105					110		
ttc	cag	gac	ttc	ttt	cct	gtg	atc	ttc	agc	aaa	gcc	tcc	gag	tac	ttg	384	
Phe	Gln	Asp	Phe	Phe	Pro	Val	Ile	Phe	Ser	Lys	Ala	Ser	Glu	Tyr	Leu		
				115					120					125			
cag	ctg	gtc	ttt	ggc	atc	gag	gtg	gtg	gaa	gtg	gtc	cgc	atc	ggc	cac	432	
Gln	Leu	Val	Phe	Gly	Ile	Glu	Val	Val	Glu	Val	Val	Arg	Ile	Gly	His		
			130					135					140				
ttg	tac	atc	ctt	gtc	acc	tgc	ctg	ggc	ctc	tcc	tac	gct	ggc	ctg	ctg	480	
Leu	Tyr	Ile	Leu	Val	Thr	Cys	Leu	Gly	Leu	Ser	Tyr	Ala	Gly	Leu	Leu		
		145					150					155					
ggc	gac	aat	cag	atc	gtg	ccc	aag	aca	ggc	ctc	ctg	ata	atc	gtc	ctg	528	
Gly	Asp	Asn	Gln	Ile	Val	Pro	Lys	Thr	Gly	Leu	Leu	Ile	Ile	Val	Leu		
	160					165					170						
gcc	ata	atc	gca	aaa	gag	ggc	gac	tgt	gcc	cct	gag	gag	aaa	atc	tgg	576	
Ala	Ile	Ile	Ala	Lys	Glu	Gly	Asp	Cys	Ala	Pro	Glu	Glu	Lys	Ile	Trp		
	175				180				185						190		
gag	gag	ctg	agt	gtg	ttg	gag	gca	tct	gat	ggg	agg	gag	gac	agt	gtc	624	
Glu	Glu	Leu	Ser	Val	Leu	Glu	Ala	Ser	Asp	Gly	Arg	Glu	Asp	Ser	Val		
				195					200					205			
ttt	gcg	cat	ccc	agg	aag	ctg	ctc	acc	caa	gat	ttg	gtg	cag	gaa	aac	672	
Phe	Ala	His	Pro	Arg	Lys	Leu	Leu	Thr	Gln	Asp	Leu	Val	Gln	Glu	Asn		
			210					215					220				
tac	ctg	gag	tac	cgg	cag	gtc	ccc	ggc	agt	gat	cct	gca	tgc	tac	gag	720	
Tyr	Leu	Glu	Tyr	Arg	Gln	Val	Pro	Gly	Ser	Asp	Pro	Ala	Cys	Tyr	Glu		
		225					230					235					
ttc	ctg	tgg	ggg	cca	agg	gcc	ctc	gtt	gaa	acc	agc	tat	gtg	aaa	gtc	768	

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Phe Leu Trp Gly Pro Arg Ala Leu Val Glu Thr Ser Tyr Val Lys Val  
 240 245 250

ctg cac cat ttg cta aag atc agt gga ggg cct cac att ccc tac cca 816  
 Leu His His Leu Leu Lys Ile Ser Gly Gly Pro His Ile Pro Tyr Pro  
 255 260 265 270

ccc ctg cat gaa tgg gct ttt aga gag ggg gaa gag tga 855  
 Pro Leu His Glu Trp Ala Phe Arg Glu Gly Glu Glu  
 275 280

&lt;210&gt; 10

&lt;211&gt; 282

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 10

Met Glu Glu Gln Glu Thr Ala Ser Ser Ser Ser Thr Leu Val Glu Val  
 1 5 10 15

Thr Leu Arg Glu Val Pro Ala Ala Glu Ser Pro Ser Pro Pro His Ser  
 20 25 30

Pro Gln Gly Ala Ser Thr Leu Pro Thr Thr Ile Asn Tyr Thr Leu Trp  
 35 40 45

Ser Gln Ser Asp Glu Gly Ser Ser Asn Glu Glu Gln Glu Gly Pro Ser  
 50 55 60

Thr Phe Pro Asp Leu Glu Thr Ser Phe Gln Val Ala Leu Ser Arg Lys  
 65 70 75 80

Met Ala Glu Leu Val His Phe Leu Leu Leu Lys Tyr Arg Ala Arg Glu  
 85 90 95

Pro Phe Thr Lys Ala Glu Met Leu Gly Ser Val Ile Arg Asn Phe Gln  
 100 105 110

Asp Phe Phe Pro Val Ile Phe Ser Lys Ala Ser Glu Tyr Leu Gln Leu  
 115 120 125

Val Phe Gly Ile Glu Val Val Glu Val Val Arg Ile Gly His Leu Tyr  
 130 135 140

Ile Leu Val Thr Cys Leu Gly Leu Ser Tyr Ala Gly Leu Leu Gly Asp  
 145 150 155 160

Asn Gln Ile Val Pro Lys Thr Gly Leu Leu Ile Ile Val Leu Ala Ile  
 165 170 175

Ile Ala Lys Glu Gly Asp Cys Ala Pro Glu Glu Lys Ile Trp Glu Glu  
 180 185 190

Leu Ser Val Leu Glu Ala Ser Asp Gly Arg Glu Asp Ser Val Phe Ala  
 195 200 205

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His Pro Arg Lys Leu Leu Thr Gln Asp Leu Val Gln Glu Asn Tyr Leu  
 210 215 220

Glu Tyr Arg Gln Val Pro Gly Ser Asp Pro Ala Cys Tyr Glu Phe Leu  
 225 230 235 240

Trp Gly Pro Arg Ala Leu Val Glu Thr Ser Tyr Val Lys Val Leu His  
 245 250 255

His Leu Leu Lys Ile Ser Gly Gly Pro His Ile Pro Tyr Pro Pro Leu  
 260 265 270

His Glu Trp Ala Phe Arg Glu Gly Glu Glu  
 275 280

&lt;210&gt; 11

&lt;211&gt; 855

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (7)..(852)

&lt;400&gt; 11

gccacc atg gag gag cag gag gct gcc tcc tcc tct tct act cta gtt 48  
 Met Glu Glu Gln Glu Ala Ala Ser Ser Ser Ser Thr Leu Val  
 1 5 10

gaa gtc acc ctg ggg gag gtg cct gct gcc gag tca cca gat cct ccc 96  
 Glu Val Thr Leu Gly Glu Val Pro Ala Ala Glu Ser Pro Asp Pro Pro  
 15 20 25 30

cag agt cct cag gga gcc tcc agc ctc ccc act acc atg aac tac cct 144  
 Gln Ser Pro Gln Gly Ala Ser Ser Leu Pro Thr Thr Met Asn Tyr Pro  
 35 40 45

ctc tgg agc caa tcc tat gag gac tcc agc aac caa gaa gag gag ggg 192  
 Leu Trp Ser Gln Ser Tyr Glu Asp Ser Ser Asn Gln Glu Glu Glu Gly  
 50 55 60

cca agc acc ttc cct gac ctg gag tct gag ttc caa gca gca ctc agt 240  
 Pro Ser Thr Phe Pro Asp Leu Glu Ser Glu Phe Gln Ala Ala Leu Ser  
 65 70 75

agg aag gtg gcc aag ttg gtt cat ttt ctg ctc ctc aag tat cga gcc 288  
 Arg Lys Val Ala Lys Leu Val His Phe Leu Leu Lys Tyr Arg Ala  
 80 85 90

agg gag ccg gtc aca aag gca gaa atg ctg ggg agt gtc gtc gga aat 336  
 Arg Glu Pro Val Thr Lys Ala Glu Met Leu Gly Ser Val Val Gly Asn  
 95 100 105 110

tgg cag tac ttc ttt cct gtg atc ttc agc aaa gct tcc gat tcc ttg 384

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Trp	Gln	Tyr	Phe	Phe	Pro	Val	Ile	Phe	Ser	Lys	Ala	Ser	Asp	Ser	Leu		
				115					120					125			
cag	ctg	gtc	ttt	ggc	atc	gag	ctg	atg	gaa	gtg	gac	ccc	atc	ggc	cac	432	
Gln	Leu	Val	Phe	Gly	Ile	Glu	Leu	Met	Glu	Val	Asp	Pro	Ile	Gly	His		
			130					135					140				
gtg	tac	atc	ttt	gcc	acc	tgc	ctg	ggc	ctc	tcc	tac	gat	ggc	ctg	ctg	480	
Val	Tyr	Ile	Phe	Ala	Thr	Cys	Leu	Gly	Leu	Ser	Tyr	Asp	Gly	Leu	Leu		
		145					150					155					
ggt	gac	aat	cag	atc	atg	ccc	aag	aca	ggc	ttc	ctg	ata	atc	atc	ctg	528	
Gly	Asp	Asn	Gln	Ile	Met	Pro	Lys	Thr	Gly	Phe	Leu	Ile	Ile	Ile	Leu		
	160					165					170						
gcc	ata	atc	gca	aaa	gag	ggc	gac	tgt	gcc	cct	gag	gag	aaa	atc	tgg	576	
Ala	Ile	Ile	Ala	Lys	Glu	Gly	Asp	Cys	Ala	Pro	Glu	Glu	Lys	Ile	Trp		
175					180				185						190		
gag	gag	ctg	agt	gtg	tta	gag	gtg	ttt	gag	ggg	agg	gaa	gac	agt	atc	624	
Glu	Glu	Leu	Ser	Val	Leu	Glu	Val	Phe	Glu	Gly	Arg	Glu	Asp	Ser	Ile		
			195					200					205				
ttc	ggg	gat	ccc	aag	aag	ctg	ctc	acc	caa	tat	ttc	gtg	cag	gaa	aac	672	
Phe	Gly	Asp	Pro	Lys	Lys	Leu	Leu	Thr	Gln	Tyr	Phe	Val	Gln	Glu	Asn		
			210					215					220				
tac	ctg	gag	tac	cgg	cag	gtc	ccc	ggc	agt	gat	cct	gca	tgc	tat	gag	720	
Tyr	Leu	Glu	Tyr	Arg	Gln	Val	Pro	Gly	Ser	Asp	Pro	Ala	Cys	Tyr	Glu		
		225					230					235					
ttc	ctg	tgg	ggt	cca	agg	gcc	ctc	att	gaa	acc	agc	tat	gtg	aaa	gtc	768	
Phe	Leu	Trp	Gly	Pro	Arg	Ala	Leu	Ile	Glu	Thr	Ser	Tyr	Val	Lys	Val		
		240				245					250						
ctg	cac	cat	atg	gta	aag	atc	agt	gga	gga	cct	cgc	att	tcc	tac	cca	816	
Leu	His	His	Met	Val	Lys	Ile	Ser	Gly	Gly	Pro	Arg	Ile	Ser	Tyr	Pro		
255					260				265						270		
ctc	ctg	cat	gag	tgg	gct	ttg	aga	gag	ggg	gaa	gag	tga				855	
Leu	Leu	His	Glu	Trp	Ala	Leu	Arg	Glu	Gly	Glu	Glu						
			275					280									

&lt;210&gt; 12

&lt;211&gt; 282

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 12

Met	Glu	Glu	Gln	Glu	Ala	Ala	Ser	Ser	Ser	Ser	Thr	Leu	Val	Glu	Val
1				5					10					15	

Thr	Leu	Gly	Glu	Val	Pro	Ala	Ala	Glu	Ser	Pro	Asp	Pro	Pro	Gln	Ser
			20					25					30		

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Pro Gln Gly Ala Ser Ser Leu Pro Thr Thr Met Asn Tyr Pro Leu Trp  
           35                          40                          45  
 Ser Gln Ser Tyr Glu Asp Ser Ser Asn Gln Glu Glu Gly Pro Ser  
           50                          55                          60  
 Thr Phe Pro Asp Leu Glu Ser Glu Phe Gln Ala Ala Leu Ser Arg Lys  
           65                          70                          75                          80  
 Val Ala Lys Leu Val His Phe Leu Leu Leu Lys Tyr Arg Ala Arg Glu  
                           85                          90                          95  
 Pro Val Thr Lys Ala Glu Met Leu Gly Ser Val Val Gly Asn Trp Gln  
                           100                          105                          110  
 Tyr Phe Phe Pro Val Ile Phe Ser Lys Ala Ser Asp Ser Leu Gln Leu  
           115                          120                          125  
 Val Phe Gly Ile Glu Leu Met Glu Val Asp Pro Ile Gly His Val Tyr  
           130                          135                          140  
 Ile Phe Ala Thr Cys Leu Gly Leu Ser Tyr Asp Gly Leu Leu Gly Asp  
           145                          150                          155                          160  
 Asn Gln Ile Met Pro Lys Thr Gly Phe Leu Ile Ile Ile Leu Ala Ile  
                           165                          170                          175  
 Ile Ala Lys Glu Gly Asp Cys Ala Pro Glu Glu Lys Ile Trp Glu Glu  
           180                          185                          190  
 Leu Ser Val Leu Glu Val Phe Glu Gly Arg Glu Asp Ser Ile Phe Gly  
           195                          200                          205  
 Asp Pro Lys Lys Leu Leu Thr Gln Tyr Phe Val Gln Glu Asn Tyr Leu  
           210                          215                          220  
 Glu Tyr Arg Gln Val Pro Gly Ser Asp Pro Ala Cys Tyr Glu Phe Leu  
           225                          230                          235                          240  
 Trp Gly Pro Arg Ala Leu Ile Glu Thr Ser Tyr Val Lys Val Leu His  
           245                          250                          255  
 His Met Val Lys Ile Ser Gly Gly Pro Arg Ile Ser Tyr Pro Leu Leu  
           260                          265                          270  
 His Glu Trp Ala Leu Arg Glu Gly Glu Glu  
           275                          280

&lt;210&gt; 13

&lt;211&gt; 321

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;220&gt;

&lt;221&gt; CDS



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&lt;222&gt; (7)..(318)

&lt;400&gt; 13

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gccacc atg ggg cac ggc cac tct tac acc acg gct gaa gag gcc gct    48
  Met Gly His Gly His Ser Tyr Thr Thr Ala Glu Glu Ala Ala
      1             5             10

ggg atc ggc atc ctg aca gtg atc ctg gga gtc tta ctg ctc atc ggc    96
Gly Ile Gly Ile Leu Thr Val Ile Leu Gly Val Leu Leu Leu Ile Gly
  15             20             25             30

tgt tgg tat tgt aga aga cga aat gga tac aga gcc ttg atg gat aaa   144
Cys Trp Tyr Cys Arg Arg Arg Asn Gly Tyr Arg Ala Leu Met Asp Lys
      35             40             45

agt ctt cat gtt ggc act caa tgt gcc tta aca aga aga tgc cca caa   192
Ser Leu His Val Gly Thr Gln Cys Ala Leu Thr Arg Arg Cys Pro Gln
      50             55             60

gaa ggg ttt gat cat cgg gac agc aaa gtg tct ctt caa gag aaa aac   240
Glu Gly Phe Asp His Arg Asp Ser Lys Val Ser Leu Gln Glu Lys Asn
      65             70             75

tgt gaa cct gtg gtt ccc aat gct cca cct gct tat gag aaa ctc tct   288
Cys Glu Pro Val Val Pro Asn Ala Pro Pro Ala Tyr Glu Lys Leu Ser
      80             85             90

gca gaa cag tca cca cca cct tat tca cct taa                       321
Ala Glu Gln Ser Pro Pro Pro Tyr Ser Pro
  95             100

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&lt;210&gt; 14

&lt;211&gt; 104

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 14

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Met Gly His Gly His Ser Tyr Thr Thr Ala Glu Glu Ala Ala Gly Ile
  1             5             10             15

Gly Ile Leu Thr Val Ile Leu Gly Val Leu Leu Leu Ile Gly Cys Trp
      20             25             30

Tyr Cys Arg Arg Arg Asn Gly Tyr Arg Ala Leu Met Asp Lys Ser Leu
      35             40             45

His Val Gly Thr Gln Cys Ala Leu Thr Arg Arg Cys Pro Gln Glu Gly
      50             55             60

Phe Asp His Arg Asp Ser Lys Val Ser Leu Gln Glu Lys Asn Cys Glu
      65             70             75             80

Pro Val Val Pro Asn Ala Pro Pro Ala Tyr Glu Lys Leu Ser Ala Glu
      85             90             95

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Gln Ser Pro Pro Pro Tyr Ser Pro  
100